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THE DISTRIBUTION AND POLLINATION TIMES OF THE IMPORTANT HAY FEVER-PRODUCING PLANTS IN THE UNITED STATES*

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IT IS axiomatic that the precise etiologic diagnosis of hay fever requires a knowledge of the pollens to which the patient is exposed; in other words, a knowledge of the identity of those plants in the patient's vicinity which are capable of causing pollinosis, their relative prevalence, the average onset and termination of their pollinating seasons, and their pollen-producing capacity. Such information for each locality is essential in correlating the patient's symptoms with the presence of his allergens, in selecting the appropriate pollens with which to perform tests, in evaluating cases clinically diagnosed as hay fever but failing to react to tests, and in choosing the proper extracts for treatment. For practical application, information about the facts mentioned is of greater importance than the variations in the quantity of atmospheric pollen grains from day to day within the season.

Few physicians, of course, have the time, the requisite botantical training, or the facilities for carrying out pollen counts or field surveys. Hence, it is necessary to rely upon pollination studies previously completed in the same, or some comparable, locale. Although the seasons vary somewhat from year to year, depending on a number of meteorologic conditions (see below), they are usually sufficiently constant for the experience of previous years to be applied clinically.

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There undoubtedly exists a great deal of unpublished data in this field. Much of it is in the hands of allergists located throughout the country, and a commendable effort has recently* been made by the Committee on a Pollen Survey of the Society for the Study of Asthma and Allied Conditions,[27] to tap this rich source of material. This investigation has not yet reached the point where it is useful to the practicing physician.

In addition, scattered throughout the medical literature are a large number of carefully performed investigations which, *in toto*, nearly blanket the United States. These, to be sure, differ widely in scope and technique, some including only a single species or one related group of plants and some reporting findings regarding only a portion of the hay fever season. Most pertain to one locale only, others cover extensive regions of the country, and still others are national or nearly national in extent. Many are based on atmospheric pollen counts by gravity or other methods, some are the result of botanical surveys, a few combine these two approaches, and still fewer correlate these findings with clinical observations. Deficiencies encountered in various articles

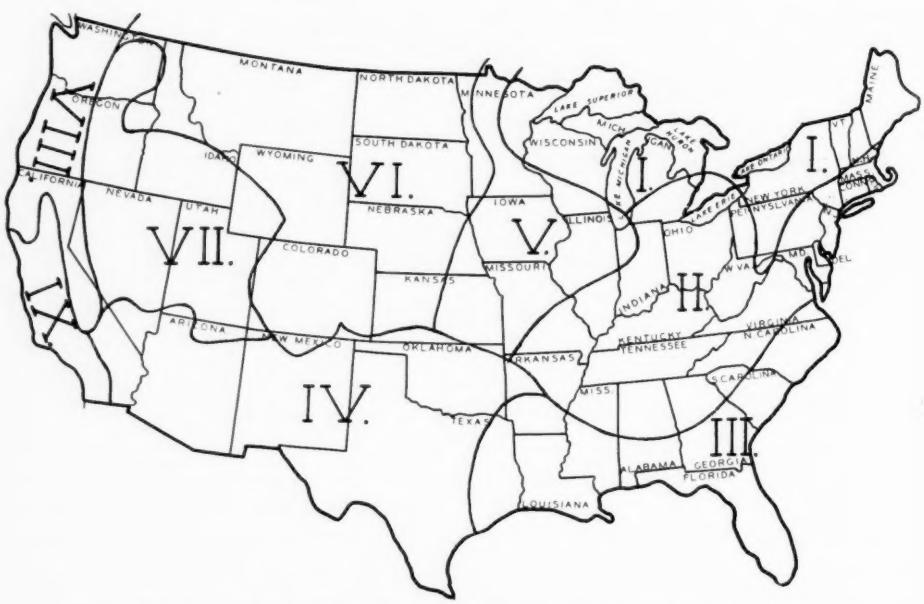

Fig. 1.—Division of the United States into nine pollination zones.

include omission of the duration of pollination and less frequently of its exact onset, grouping of several species into rough "seasons" rather than giving the separate dates, and inadequate identification of plants by ambiguous common names. Many fail to state the relative importance of each plant, or where given, to mention whether it is based on pollen counts, prevalence, abundance of pollen production, or the frequency of positive skin reactions. A common failing is the assumption, unsupported by actual determinations or any apparent scientific evidence, that the findings in a given city are characteristic of a whole state or even larger area, such as New England, the Middle Atlantic States, or the South.

*At the time this paper was undertaken and started, no such study had been begun.

Despite all these shortcomings, which, of course, by no means apply to all the reports, a great deal of useful information can be gained. And inasmuch as the data for one community can properly be considered to hold for at least a limited portion of the surrounding territory, all these observations can be compared and combined to give a representative, over-all picture of the pollen situation in the United States.

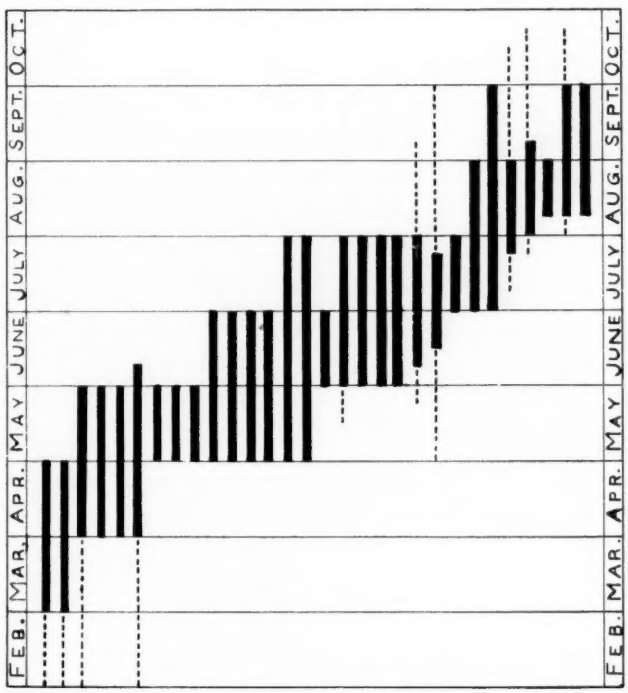

Fig. 2.—Pollination calendar for Zone I. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *I*, Occurs in western portion of the zone only.

It is a botanical truth that probably no two species of plants precisely correspond in their distribution, range, or region of maximum growth.[1] Moreover, depending on the nature of the soil, the elevation, and a multitude of meteorologic factors, the dates of onset and cessation of pollination vary from place to place in the same area. Hopkins[2] has pointed out that, other conditions being equal, the variation in time of occurrence in temperate North America is at a general average rate of four days to each 1 degree of latitude, 5 degrees of longitude, and 400 feet of altitude, later northward, eastward, and upward

in spring and early summer, and the reverse in late summer and autumn. The average advance of the season per day is seventeen miles northward, sixty-two miles eastward, and 100 feet upward.

Nevertheless, for practical and clinical purposes, rather large districts can be considered to contain a certain type of flora with pollination seasons which, with some variation, are by and large uniform for that area. This is all the

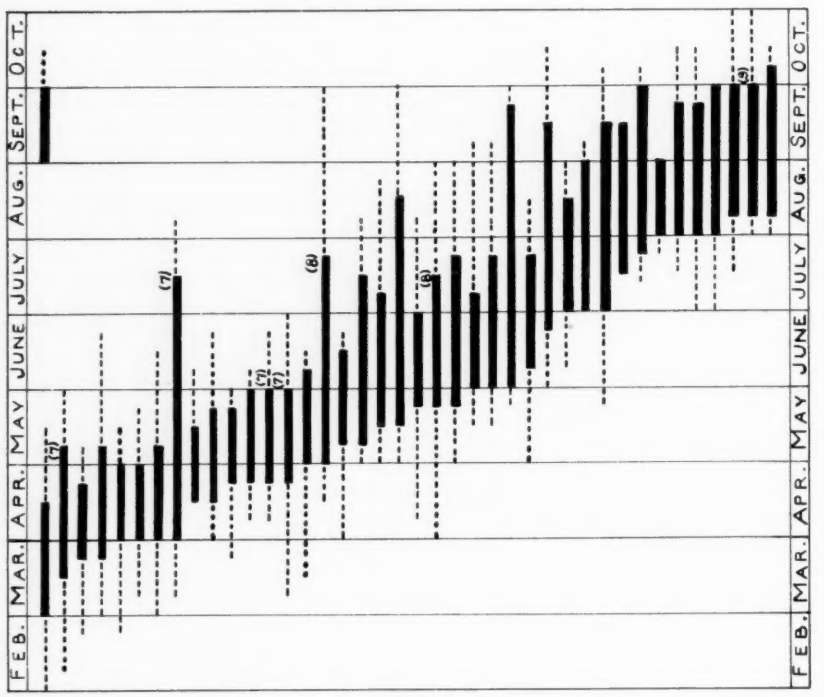

Fig. 3.—Pollination calendar for Zone II. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, Much less common in southern portion of zone; *2*, less common in northern portion; *3*, also *Poa compressa* (Canadian blue grass) in northern portion; *4*, also *Plantago major* (common plantain), but less common; *5*, occurs in southern portion only; *6*, occurs in western portion only; *7*, pollinates one month earlier in southern portion; *8*, sometimes pollinates in January or February in southern portion; *9*, pollination commences somewhat later in southern portion.

more true in that the important hay fever-causing pollens can be carried by the wind for considerable distances, such atmospheric pollution tending to minimize the influence of local differences in plant life. It has been customary to divide the country into districts according to state boundaries or the usual geographico-economic units. A moment's thought will show that this cannot be correct for the present instance, since neither the plants themselves nor their air-borne pollens are any respectors of state borders. Even where states are bounded by rivers, the same flora is practically always established on both

banks. Hence, we have more logically resorted to the botanical-vegetational areas painstakingly delineated by Livingston and Shreve.[1] It was found necessary to modify their map to accord with more recent publications and with the

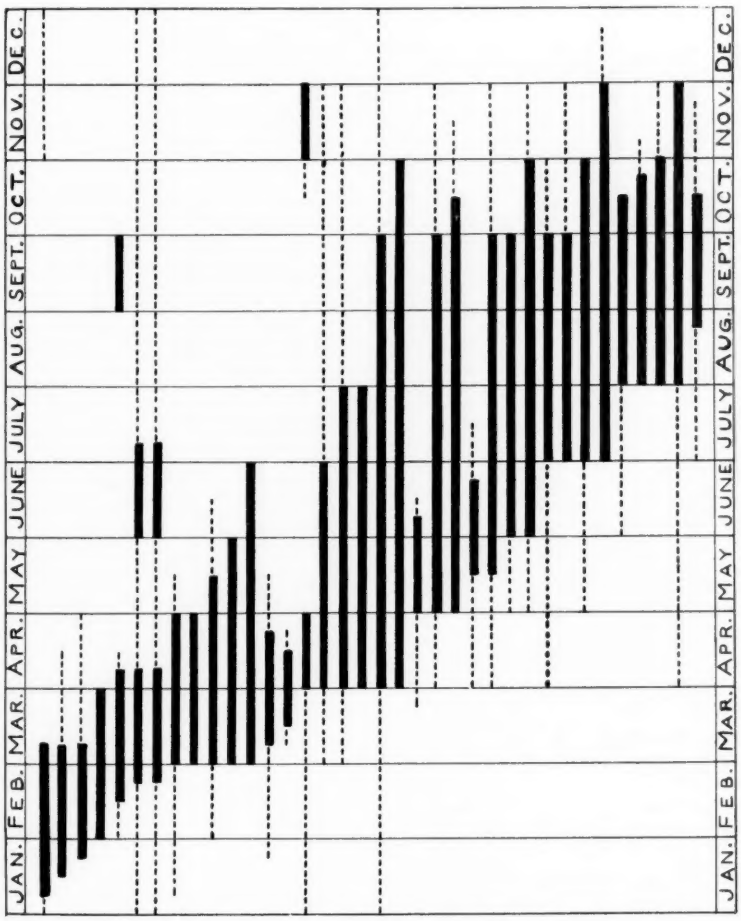

Fig. 4.—Pollination calendar for Zone III. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, Occurs in Florida only; *2*, occurs in southern Florida only; *3*, occurs chiefly in western portion—none in Florida; *4*, none in eastern Florida.

fact that we are solely concerned with plants capable of producing hay fever rather than with all the flora. Fig. 1 presents a division of the United States into nine zones according to these principles.

The literature yielded 166 articles containing usable information. Of these, forty-two covered the entire country, or at least large areas, as regards one or more species, and these are given first in the references. Parenthetically, it may

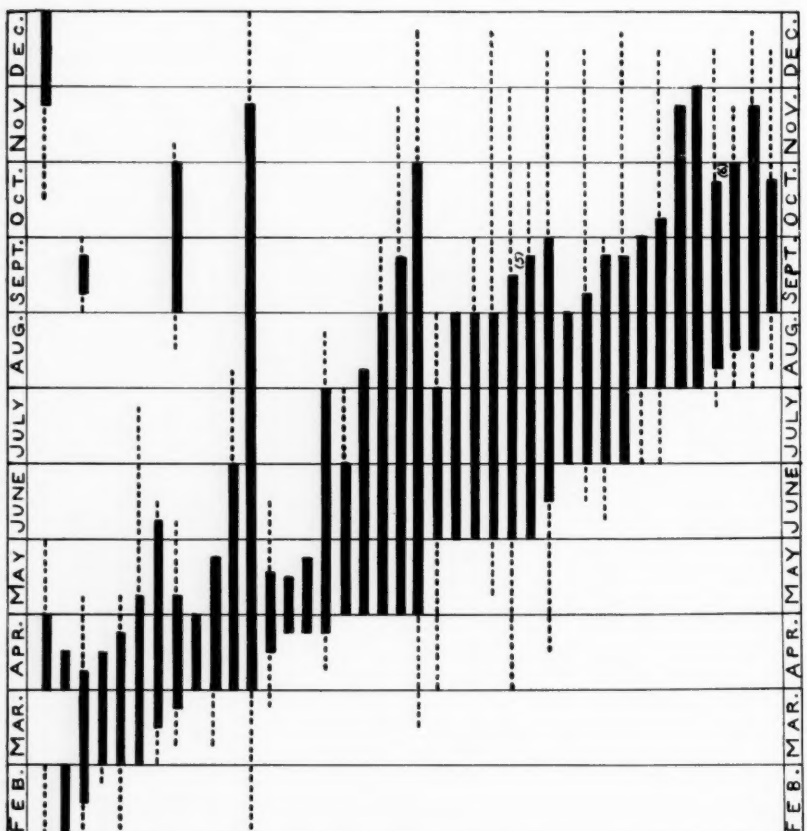

Fig. 5.—Pollination calendar for Zone IV. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. 1, Less common in western portion; 2, occurs in western portion only; 3, less common in eastern portion; 4, none in western portion; 5, pollinates perennially in protected areas; 6, pollinates earlier in extreme western Texas.

be noted that in six of them attention was confined to the West;[4, 20, 31, 36-38] in four, to the South;[8, 16, 29, 45] in four, to the Midwest;[7, 9, 22, 23] and in three, to New England and the Middle Atlantic States.[26, 39, 40] The remainder of the

articles are based on local studies or concern at most a state and are so listed. (In a few instances, portions of adjoining states were given consideration. These are entered only under the state primarily represented.) The bibliography also contains the references for Canada and Alaska.

By combining and, so to speak, "averaging" for each zone, the score or more surveys done in separate localities by various methods in different years, it is felt that a comprehensive view is obtained, which is, in some respects, possibly more accurate than the original studies themselves. The results of this method are given in Figs. 2 to 10, which constitute a pollination calendar for each of the nine zones.

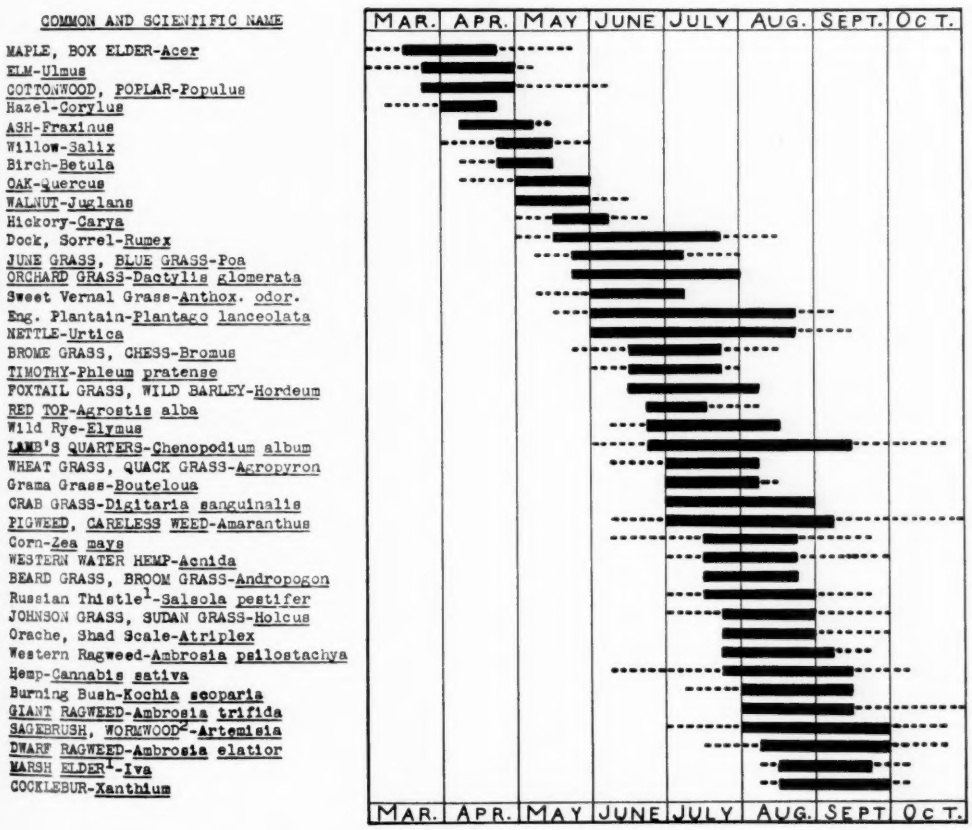

Fig. 6.—Pollination calendar for Zone V. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, None in Milwaukee, Wis.; *2*, very little in Omaha, Neb., and Milwaukee, Wis.

It must not be thought that all the plants mentioned in the literature are included in the figures. Only those of considerable importance were selected. As a matter of fact, references to nearly 300 species of weeds alone are to be found, plus hundreds of grasses and trees. The main pollination times are given in a heavy, unbroken line. This tends, in most instances, to be somewhat longer than the actual season at any one place in any one year because of the totaling of the commoner annual deviations, the combination of observations

from widely separated localities, climatic variations, differences in the time of planting of cultivated species, and other factors. This is especially true of the spring and early summer seasons rather than of late summer and fall.

It should also be pointed out that many plants pollinate sporadically both before and after the principal season, that local circumstances may influence the dates over a limited area, and that unusual weather conditions may correspondingly accelerate or delay pollination. All this is signified in the calendars by

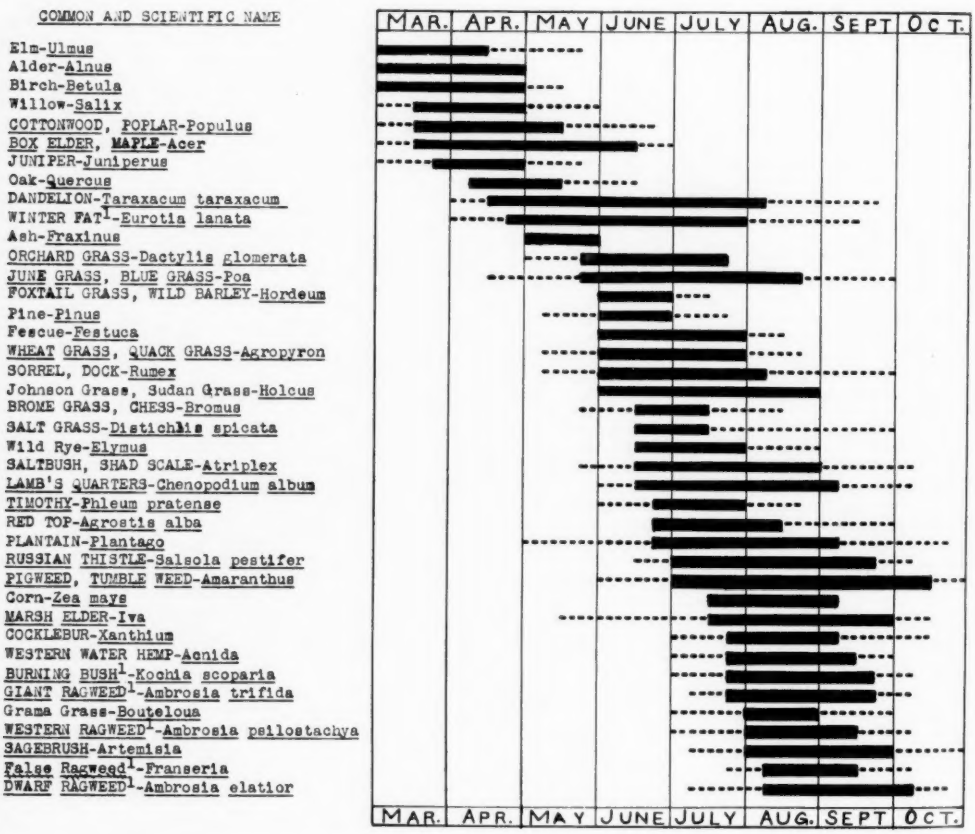

Fig. 7.—Pollination calendar for Zone VI. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, None in western Colorado, western Montana, or northern Idaho.

the finer, interrupted lines. Finally, the various trees, grasses, and weeds have been graded, according to their frequency and importance, into three groups: the major causes of hay fever, those of secondary significance, and those of lesser consequence. This, likewise, is indicated in the figures. Needless to say, other plants can, less often, be responsible for occasional cases of pollinosis, either as a result of unusual exposure or of extraordinary weather conditions. For localities near the boundary of a zone, it is suggested that the calendar for the adjacent zone also be consulted.

Naturally, certain sources of error are inherent in pollen surveys, whether by atmospheric count or botanical observation, and apply equally to this study, in so far as it is employed in any one locale.

In the first place, the beginning as well as the end of the pollination period depends, from year to year, on meteorologic factors, particularly the range of temperature, the percentage of sunshine, and the amount of precipitation. Thus, an especially warm spring and summer with heavy rainfall will appreciably advance the date of the first blooming, whereas a cold and dry preseason will delay it. The pollen calendar cannot do more than give the average dates.

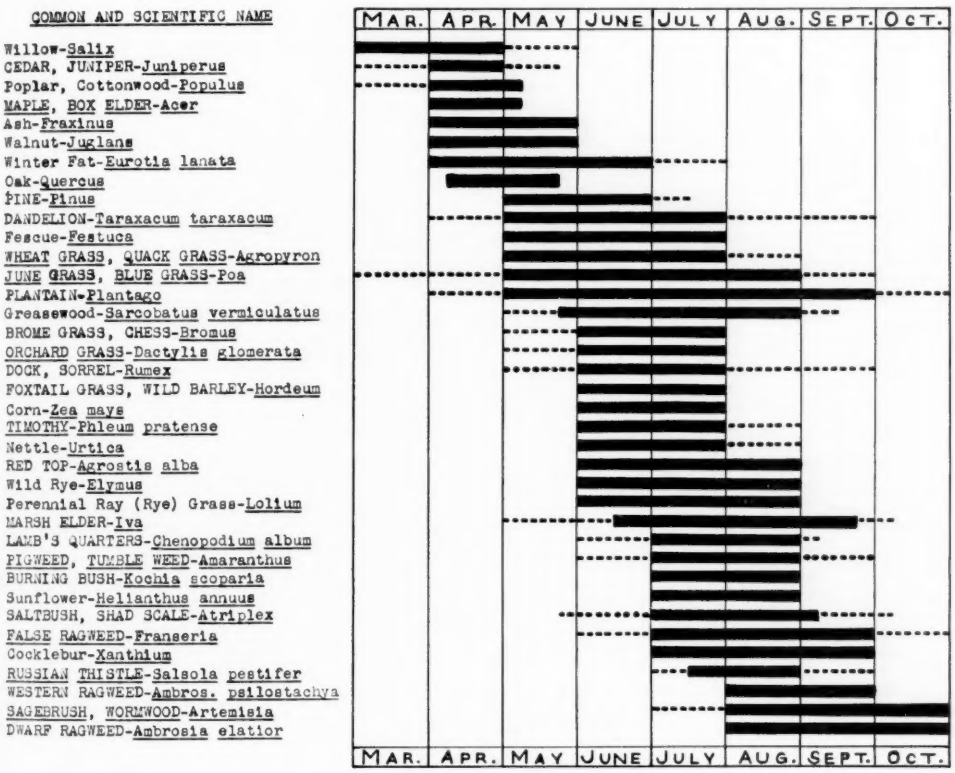

Fig. 8.—Pollination calendar for Zone VII. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively.

Fig. 11 shows how, under certain circumstances, the flowering can be hastened or retarded as much as two to four weeks. It will be seen that the onset of pollination of orchard grass (*Dactylis glomerata*) was found to vary in six consecutive years in one place from May 2 to May 29.

In the second place, extraordinary weather conditions can cause pollens which, under normal circumstances, are relatively unimportant in a given area to become major factors quite suddenly. Thus Feinberg and Durham[97] reported that in the Chicago area in 1934, unlike other years, the pollens from certain trees and from certain chenopodiales (for example, Russian thistle) were the principal allergizing agents. Duke[120] made the opposite observation that pollens which ordinarily were abundant in Kansas City, Mo., were either absent or nearly so in a season with unusual weather conditions.

Furthermore, in isolated cases, due primarily to uncommon conditions of exposure, individuals may be allergized by the pollen of trees, flowers, and

bushes which only very rarely cause hay fever. For example, Dutton[149] and Phillips[58] showed that in the fields near El Paso, Texas, and Phoenix, Ariz., respectively, where the common sugar beet (*Beta vulgaris*), a member of the family Chenopodiaceae, is intensively cultivated, some cases of hay fever are due to its pollen, which is shed from early May to mid-June. Present plans to augment greatly the acreage devoted to this plant may well lead to an increase

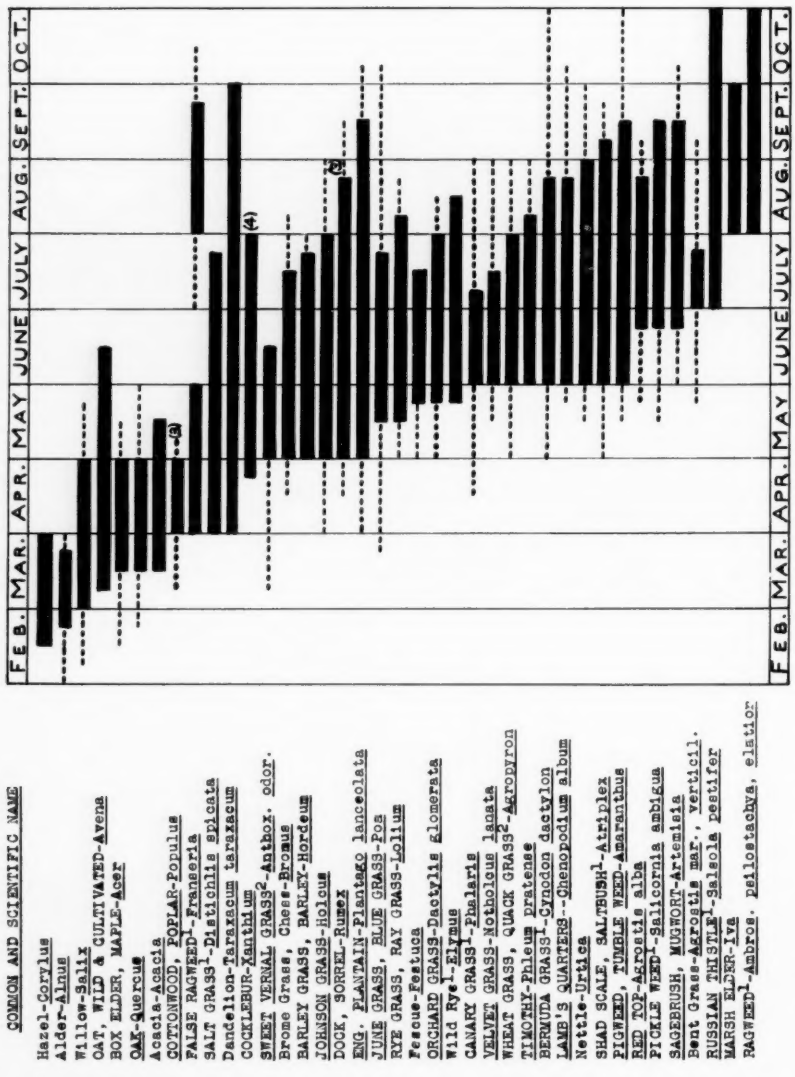

Fig. 9.—Pollination calendar for Zone VIII. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type, respectively. *1*, None in northern portion; *2*, much less common in southern portion; *3*, pollinates one month earlier in southern portion; *4*, pollinates later in extreme southern portion.

in the number of such cases. McMinn and Graham[65] found that the pollen of the mirror plant (*Coprosma baueri*), an ornamental shrub widely planted in California and pollinating from the beginning of April to the end of June, yields positive skin tests in a considerable group of patients with hay fever. Langley[140] reported an unusual case of hypersensitiveness in a nurseryman to the pollen of an exotic plant, *Piqueria trinervia*, due to hothouse exposure through the winter. One of us (E. U.) encountered two exceptional cases in which hay fever

was evoked by the pollens of tree of heaven (*Ailanthus glandulosa*) and horse chestnut (*Aesculus hippocastanum*), respectively. Mention should also be made here of the possibility of allergy to insect-borne pollens under appropriate conditions.

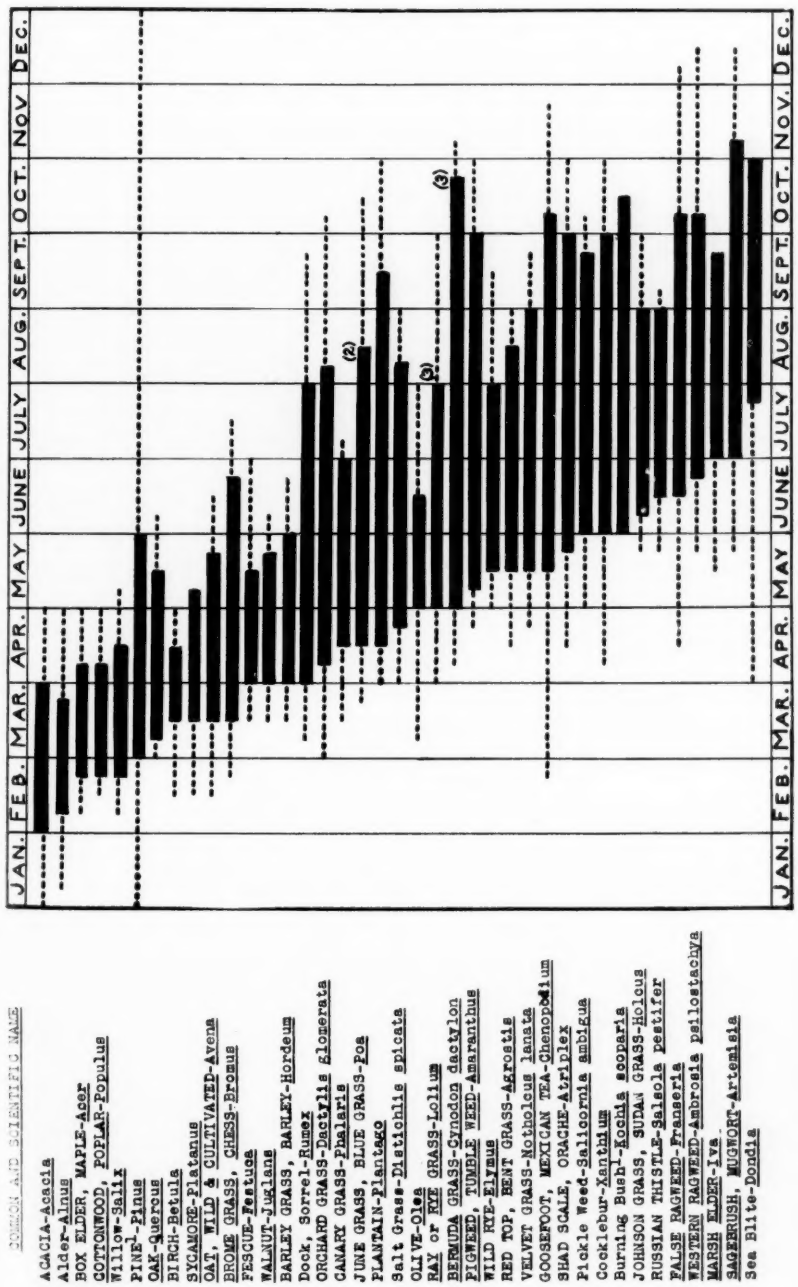

Fig. 10.—Pollination calendar for Zone IX. Most important pollens, those of secondary importance, and the least important are indicated by underscored capitals, capitals, and capital and lower case type respectively. *1*, Less common in southern portion; *2*, pollinates perennially in certain areas; *3*, pollinates perennially in southern portion.

A third important source of error lies in the fact that, even within a relatively small area, differences in the elevation above sea level play a significant part in advancing or delaying the date of first pollination by a month or more.

This applies, for example, to the variations in pollination time in a valley and the adjacent mountains. As mentioned above, for each 400 feet of altitude, the delay amounts to about four days. It has also been observed (Mullin[77]) that the nature of the flora present varies greatly with increasing altitude.

Despite all these obvious and inevitable faults, such tabulations of data as the pollen calendars are certainly of great general value to the physician as a guide in the diagnosis and treatment of hay fever.

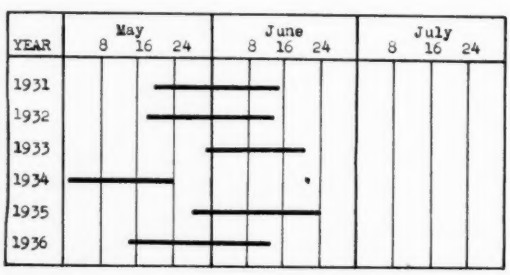

Fig. 11.—Pollination times of a single plant (orchard grass—*Dactylis glomerata*) in six successive years. Note the marked variation (as much as four weeks) in the onset and cessation of pollination in certain years.

It must be admitted that some of the plant prevalences and pollination dates presented here are not always in accord with the accepted ideas. It can only be said that they represent a careful, impartial evaluation of the published material after the elimination of the apparently less reliable observations. This accumulated information cannot be considered as final but could well be supplemented by further surveys, preferable on a nationwide scale and according to a uniform technique. The desirability of this and the locations in which it is most needed will be discussed in a separate article.[3]

SUMMARY

The necessity for a thorough knowledge of the distribution, prevalence, and pollination dates of the trees, grasses, and weeds which cause pollinosis is well known.

For this purpose, the United States has been divided into nine zones, according to botanical considerations. The plants which most commonly produce hay fever, their pollination times, and their relative importance are presented in nine pollination calendars, one for each zone.

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SURFACE FILMS FORMED BY BLOOD PLASMA AND SERUM OF PATIENTS WITH CHRONIC ARTHRITIS*

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INTRODUCTION

PATIENTS with chronic arthritis present deviations from normal in respect to both the cellular and noncellular components of the blood. Anemia[1] is common, and moderate leucocytosis and relative increments in the proportion of aged neutrophiles are frequent. Few significant alterations from normal have been found with respect to the fasting levels of substances having small molecular weights and high solubility in water, such as sugar, urea, and electrolytes.[2] While none of the abnormalities of blood thus far described are pathognomonic for the rheumatic syndrome, they are significant in that they reflect evidence of systemic disturbances. Among newly available procedures which might be expected to throw additional light on the nature of the pathologic alterations in the fluid tissues of arthritis is one which depends upon the formation of surface films by the substances known to be present in the blood in altered amounts. Abnormal levels of globulin and albumin in the blood of certain clinically active atrophic arthritic patients have been demonstrated in this[3] and other laboratories.[4, 5] These deviations are associated with decreased suspension stability of the erythrocytes and with gelation of plasma upon the addition of formaldehyde.[6] Blood sera from comparable cases show agglutinins for hemolytic streptococci.[7]

Purified proteins and lipoids possess the quality of spreading on the clean surface of an aqueous substrate to form thin films. Such films have been extensively studied by Adam,[8] Langmuir,[9] Langmuir and Schaefer,[10, 10a, 10b] Harkins,[11] Gorter,[12, 12a] Blodgett,[13] and many others. A recent review by

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Gorter[14] includes a comprehensive bibliography covering particularly studies on films produced by compounds of biologic interest. The surface films produced by various substances exhibit characteristic qualities with respect to extent, viscosity, compressibility, thickness, and structure. Such films may be formed by remarkably small amounts of material. Under appropriate conditions certain materials spread spontaneously to form films which are only one molecule in thickness. The small quantities required for observation and the characteristic qualities exhibited by physiologically important compounds, such as lipoids and proteins, suggest the possibility that the procedures developed in this field might be well adapted to the study of body fluids in health and disease.

The present report presents results of an exploratory survey made to ascertain the extent to which methods of surface film study are adaptable to clinical materials, especially as presented by patients with rheumatic diseases. A relatively simple technique based upon more elaborate methods used in the study of surface films formed by purified oils, fatty acids, other lipoids, and proteins has been devised; this reveals differences between blood fluids obtained from certain rheumatic subjects and from control subjects. A completely satisfactory interpretation of the significance of the differences encountered would require an extended and exhaustive analysis of each component fraction of the complete fluid. Such an analysis is beyond the scope of the present survey. The data available, however, disclose a new physical index of systemic deviations from normal in the arthritic patient. It appears probable that comparable deviations occur in other clinical conditions, and application of the technique described is not limited to rheumatic disorders.

CLINICAL MATERIALS

Patients from whom blood specimens were obtained for study were in the dispensary, ward or private services of the Abington Memorial Hospital, primarily because of rheumatic complaints. The general class of rheumatic disease, together with relevant records regarding the clinico-pathologic picture, is indicated with individual data on the surface films formed by the specimens.

APPARATUS

The apparatus and general procedure represent an adaptation of methods described by Langmuir.[10] Details of construction are presented, not only to indicate fully the conditions under which data were secured, but also to show that semiquantitative results can be secured even though commercial models of surface film balances or elaborate facilities for the construction of instruments are unavailable. A rectangular tray of brass was made by attaching tapped square rods (⅜ in.) with screws to the edges of a brass plate 30 × 8 × ⅛ in. A second rectangular enclosure was made by attaching similar square rods within the first at a distance of ¼ in. from the sides and 1 in. from the ends. The second enclosure provides a water bearing which automatically guides a floating barrier used in the preparation of plates with built-up films. Blodgett[13] has demonstrated that layers of barium stearate can be used to afford a

measure of the thickness and homogeneity of films of unknown composition. The tray was fitted with a well 1 in. in diameter and 3 in. deep, 5 in. from one end of the tray. The inside of the tray was painted with black Duco, which serves several purposes; viz., seals the edges, makes materials floating on the surface of the water readily visible, and prevents contamination of the substrate by metallic ions from the tray. Before use, the inside of the tray and the edges were coated with paraffin in order to make them hydrophobic.

To facilitate measurements of area, one edge was marked off in ½ centimeter lengths. In order to provide an index of the forces exerted by films a chain balance was suspended six inches from one end of the tray. This balance consisted of a beam (made from bicycle wheel spokes), a knife edge (one-half of a safety razor blade), supported on a bearing made by depressing angles in a glass rod. Lateral forces exerted by the film were transmitted to the beam by way of a vertical extension from the beam loosely attached to a float of mica, 5¼ in. by ¾ in. In order to prevent movement of the films around the edges of the balance float and at the same time to permit the necessary freedom of motion, the following arrangement was devised. The float was fitted at each end with thin steel strips with equilateral notches, ⅛ in. Similarly notched strips (segments of safety razor blades) were attached to the top of the inner edge of the tray. A floating adaptor was constructed by fastening a piece of mica (⅜ in. by ⅛ in.) at right angles to a strip of thin steel ½ in. × ¼ in. The latter was notched by a square ⅛ in. by ⅛ in. to accommodate the mica. This adaptor was coated with paraffin and placed between the edge of the tray and the balance. While permitting movement of the float of the balance, the adaptor prevents passage of any material on the surface water around the edge of the balance. Pressure exerted by the films being transmitted to the beam can be counterbalanced and hence estimated by raising or lowering the free end of the chain. The relative length of the chain supported by the beam provides an index of pressures exerted on the float. The relative magnitude of the forces encountered were estimated by calibrating the length of chain in terms of the known forces exerted by films of oleic acid (29.5 dynes) and castor oil (15.0 dynes). The construction and appearance of the apparatus are shown in Figs. 1 and 2.

PROCEDURE

After filling the tray with distilled water to a level of about 1/16 in. above the edge, the surface was swept clean by a sliding paraffin-coated chromium-plated brass rod from the balance toward each end of the tray. Materials floating on the surface were thus confined to the ends of the tray. Such sweeping was repeated four or five times. As a result of these measures the surface of the water was made essentially free from gross contamination. On standing, under conditions prevailing in our laboratory, a film of essentially constant size accumulated on the working area, as shown by moving a swing of the balance from a poised position when a barrier was advanced toward the float. Unless the area of this blank exceeded 10 sq. cm. it was assumed that there was no significant source of contamination in the tray. As a result of

observations on the blood plasma of about thirty arthritic subjects, it became apparent that the materials in approximately one cubic millimeter of plasma produced films which could be handled within the working area. In view of the difficulties involved in the direct discharge of this small volume of fluid from available measuring apparatus, it was necessary to dilute the sample. One c.c. of blood plasma or serum was diluted with 4 c.c. of 0.85 per cent sodium chloride solution. Five cubic millimeters were discharged from a differential pipette. While some encouraging results were secured by the direct transfer of a droplet of the diluted sample from a pipette to the surface of

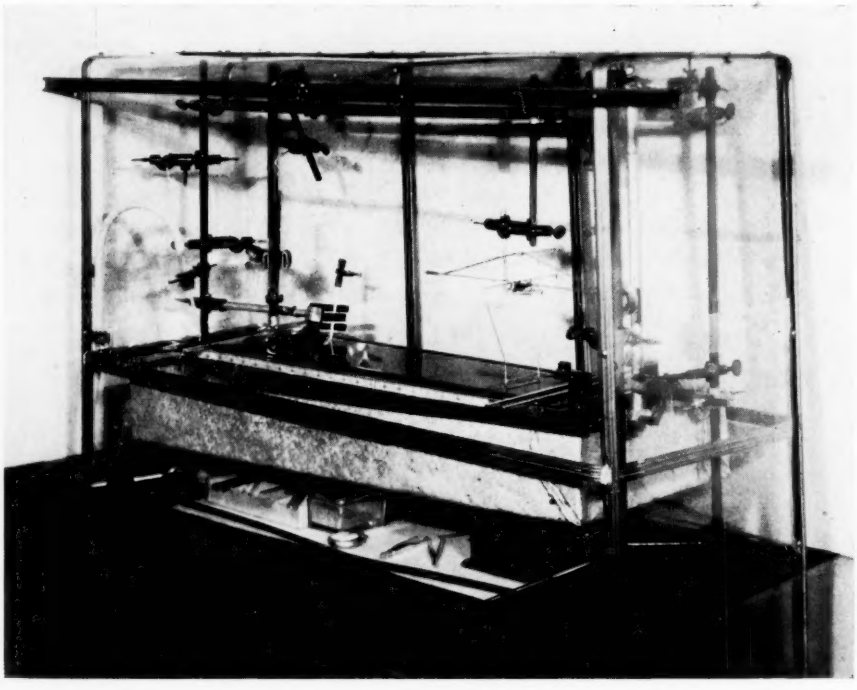

Fig. 1.—Photograph of surface film balance showing construction and arrangement of parts.

water, reproducible results were not secured with satisfactory uniformity. It was learned that more nearly reproducible data were obtained by the intermediate transfer of the diluted specimen to a metal plate. The plate held in a vertical position was then lowered cautiously through the prepared surface of water until the specimen made contact with the latter. The film-forming materials spread to the free surface of the water. After standing for five minutes the plate was dipped several times in order to insure the transfer of all of the specimen. Whether the improved reproducibility of results was actually due to this step or to increased skill in manipulation has not been established. The data presented here were secured by this procedure. The quantity of film-forming material so introduced was insufficient to cover the entire area of the tray. The films could be moved on the surface by slowly advancing the barrier toward the balance until the latter moved away from the poised po-

sition. The barrier used for maneuvering the film was fitted with a glass slide cemented on at right angles, thus functioning as a T-square. The longitudinal extent of the film was then noted, together with the weight required to counterbalance the force exerted by the film. Successive decrements of area were made by advancing the barrier by steps of ½ cm. This was followed by mak-

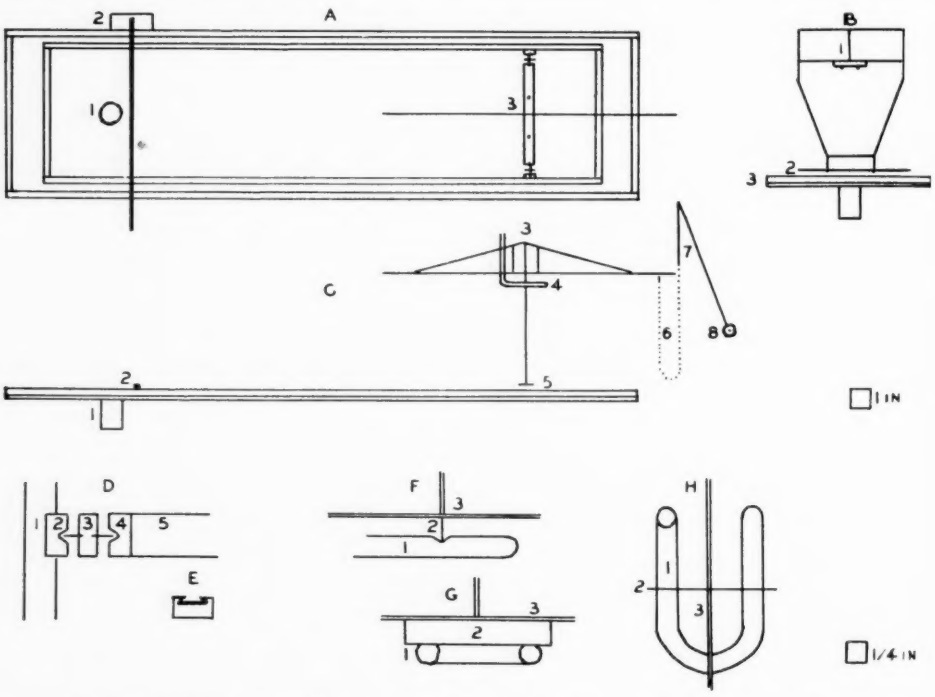

Fig. 2.—Line diagrams showing construction of surface film balance. Description:
A. Top view of balance: *1*, well; *2*, movable barrier; *3*, balance.
B. End view of balance: *1*, beam; *2*, float; *3*, tray.
C. Side view of balance: *1*, well; *2*, movable barriers; *3*, beam; *4*, support and bearing; *5*, float; *6*, chain; *7*, thread; *8*, rod windlass.
D. Top view showing attachment of float to edge of tray: *1*, edge of tray; *2*, thin steel notch; *3*, adapter; *4*, thin steel notch; *5*, mica float.
E. End view of adapter.
F. Side view of bearing: *1*, glass bearing; *2*, knife edge; *3*, beam.
G. End view of bearing: *1*, glass bearing; *2*, knife edge; *3*, beam.
H. Top view of bearing: *1*, glass bearing; *2*, knife edge; *3*, beam.

ing corresponding adjustments of the balance. Force-area charts were constructed by plotting the area in square centimeters as abscissa and the force in dynes as ordinate. The force-area diagrams so produced by different specimens were then compared by inspection.

RESULTS

In order to indicate the range of values secured by repeated determinations with the same specimens of blood, representative data are shown in Fig. 3. While the points in these instances are not identical, variations are sufficiently close to permit a satisfactory comparison of areas covered at any convenient reference pressure. Data secured with other samples were at least equally

satisfactory. It may be observed that a significant difference in area appears between the films produced by plasma and serum of the same blood. Differences of corresponding magnitude appeared between fresh and old plasmas from rapidly sedimenting bloods.

Data showing the range of representative data encountered among specimens from arthritic patients are shown in Fig. 4. While inspection reveals some differences in the slopes of the force-area curves, these are too small to be considered significant; however, the range of variations of film size are too great to be referred to variations in technique alone and must be regarded as real. Variations in area from 150 sq. cm. per 1 c.mm. plasmas to 545 sq. cm. per 1 c.mm. plasma were encountered.

Curve VC represents the force-area diagram produced by a surface film formed by plasma from a patient with the most extensive and severe active atrophic arthritis in the entire series. This patient died one month later with a pathologic diagnosis of amyloidosis with atrophic arthritis. At the time of the last observation the total protein was only 4.4 Gm. per 100 c.c. Curves CD and DL represent data from patients with active arthritis, each of whom presented sedimentation rates of 1 mm. per min., but with a total protein of 7.2 Gm. per 100 c.c. Curve PP represents another normal control subject with a normal sedimentation rate. MP represents a case of hypertrophic arthritis which is intermediate between the normal subjects and atrophic arthritic patients.

DISCUSSION

Although the initial and final portions of the force-area curves of the several films are variable, the middle portion approximates a straight line. For limited purposes it may be assumed that extrapolation of this segment of the curve to the base line represents the effective area covered by the film at "zero" pressure, or more accurately, a suitable reference pressure. Obviously, any other selected pressure within the limits of 13 to 15 dynes could be used as a reference point. It is conceivable that the first swing of the balance is produced by an extended portion of the film only or a "gaseous" film. For this reason an appreciable amount of compression is essential in order to provide assurance that the apparent area of the tray is covered by a substantial film. A constant increment of pressure with decreasing area may be regarded as indicating the formation of a reasonably stable film. The final stage, characterized by a relative decrease in pressure with diminishing area, may be attributed to collapse, folding or submersion of the film. The possibility that some components of the film are forced into solution cannot be disposed of by data at hand. The release of pressure after compression in excess of 15 dynes did not uniformly yield a film equal in area to that of the original.

It is probable that the film formed by blood fluids is composed of several substances. Certain components of blood are known to form surface films when used alone. Lipoids, i.e., fatty acids and sterols, and proteins are known to possess film-forming properties. Furthermore, surface films of mixed composition have been prepared from several of the aforementioned classes of

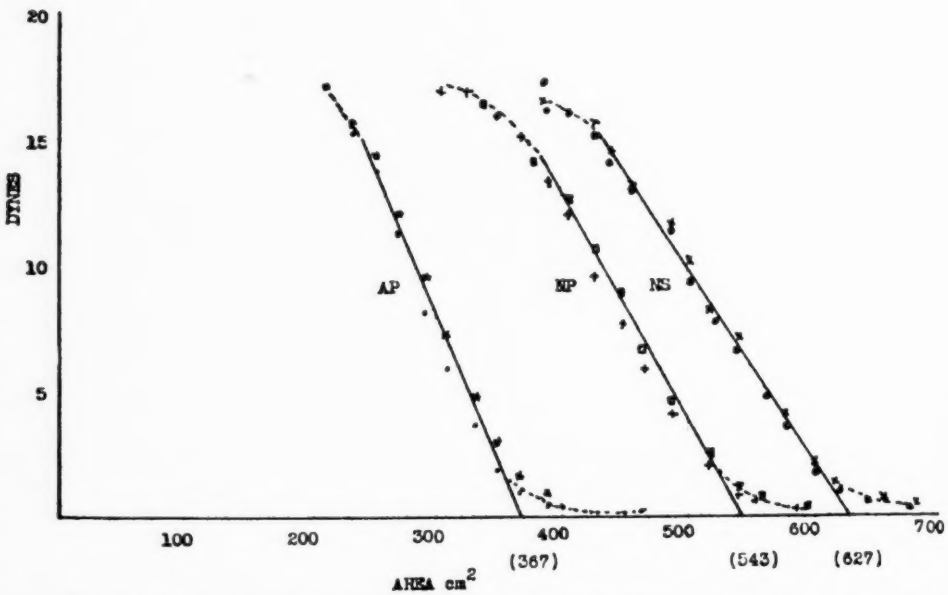

Fig. 3.—Chart showing comparative data in repeated determinations of the pressures exerted by surface films when confined to progressively decreasing areas. These films were produced from one cubic millimeter of plasma or serum from normal and arthritic subjects. AP, atrophic arthritic plasma; NP, normal plasma; NS, normal serums. From left to right, subjects: DR, WS, WS.

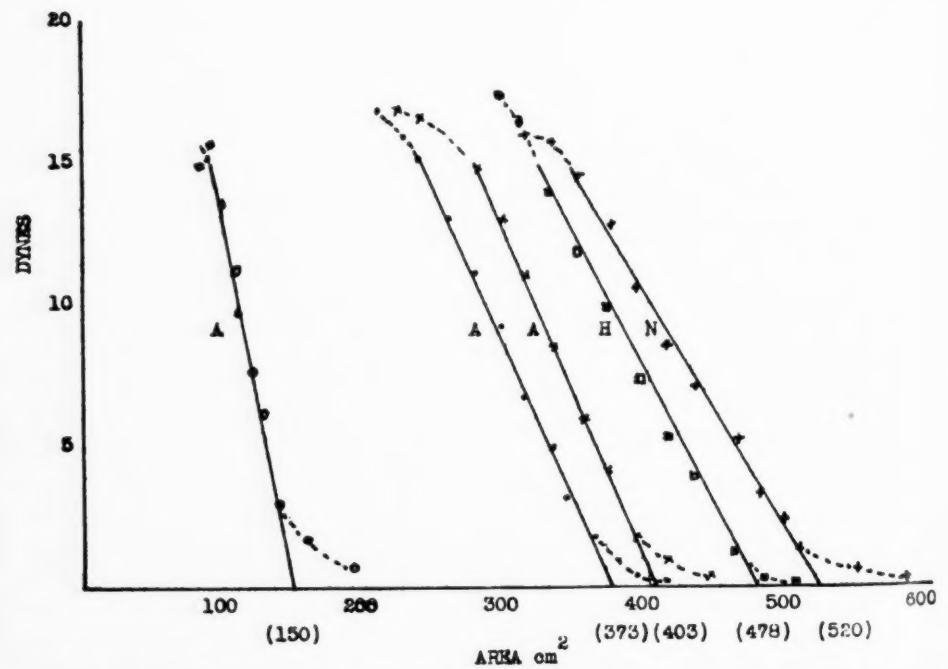

Fig. 4.—Chart showing the range of force-area curves of films produced by one cubic millimeter of blood plasma from representative arthritis: A, atrophic; H, hypertrophic; N, normal. From left to right, subjects VC, JD, DL, MP, PP.

compounds. A full account of the role of the individual compounds of the surface film formed by blood serum is beyond the scope of the present exploratory study.

The values recorded are not to be regarded as absolute and might vary with different techniques. There can be little doubt, however, as to the reality of the relative differences observed. The films formed by materials from blood serum are probably at least 25 per cent larger in area than can be accounted for by the proteins contained in them, even on the assumption that the latter are present in their most extended state. It is conceivable, however, that modifications in spreadable qualities may occur during the process of isolation of the proteins.

Another point of interest is the apparently larger areas of the films produced by serum as compared with those of plasma. This is particularly noteworthy inasmuch as fibrinogen, a spreadable material, is absent from the serum. This suggests that the reactions leading to the formation of fibrin from fibrinogen liberate materials with spreadable qualities. Determinations of film areas produced by a given plasma repeated at daily intervals revealed substantial increments. In vitro ageing of certain plasmas apparently involves a liberation of film-forming material. It is further relevant to call attention to the possible correlation of increased spreadability of serum and the suspension stability of the erythrocytes. Cells which settle rapidly in pathologic plasmas settle much more slowly in the corresponding serum and in aged plasma. These facts suggest that serum and aged plasma increase the suspension stability of red cells because of the relatively large amounts of film-forming substance as compared with the quantities present in fresh plasma. An alternative explanation based upon dilution of plasma by fluid from cells due to excessive salt concentration; viz., sodium oxalate, is not to be completely dismissed although this influence appears to be minimal.

While it may be considered possible that differences in the physical qualities of the film formed by blood serum with a high level of globulin might differ from that containing normal globulin concentrations, data presented by Gorter[11] would appear to minimize this probability. Several blood proteins, although widely different in general physical qualities, spread to form films of essentially the same magnitude. Thus both pseudoglobulin and fibrinogen form films to the extent of one square meter per milligram. Serum albumin which differs markedly from both of the former, in respect to molecular weight, electrophoretic mobility and solubility, spreads to form a film only 2 per cent less in area. Apparently such differences as may be presented by pathologic specimens are not directly related to differences in the protein components alone.

According to press reports Langmuir[15] has patented the use of the variable thickness of surface films for the diagnosis of disease. For this reason preliminary observations on the thickness of these films by the method of Blodgett,[13] although initiated, were not further pursued in the present studies.

The adaptability of the procedure to the study of clinical materials has been reasonably well established, and the method affords a practical means

of studying extremely small quantities of biologic fluids. Comparison of the spread of a mixed film with the sum of the areas of its components may afford a means of detecting otherwise unknown substances in body fluids.

A comparison of data on various arthritics with controls suggests that patients showing the greatest general systemic involvement and clinical activity present the greatest departures from normal insofar as areas of surface films are concerned. Such deviations from normal are in the direction of lowered rather than increased areas. It appears likely that certain nonarthritic patients, particularly those with lowered levels of plasma albumin, may exhibit a similar departure from normal. For this reason, deviations from normal areas of surface films are not to be regarded as satisfactory solitary indices of the arthritic process per se. Regardless of the precise interpretation to be placed upon the surface films from blood plasma of the arthritic patients, it is evident that the illness of the rheumatic patient involves more than disturbance in joints.

SUMMARY

A brief survey of an adaptation of procedures employed in the study of surface films directly to clinical materials reveals suggestive possibilities. A semiquantitative technique for the production and study of films on the surface of water from small amounts (one cubic millimeter) of blood serum and plasma has been developed. Differences have been observed with respect to the areas of films produced by equivalent volumes of normal and pathologic specimens. The areas of film formed by blood fluids from patients with rheumatic disorders are frequently smaller than those produced by blood fluids of normal subjects. One cubic millimeter of normal plasma produced films with areas of 550 sq. cm., whereas the same quantity of plasma from certain severely ill atrophic arthritic patients produced films ranging from 150 to 450 sq. cm. Films produced by plasma from less acutely ill arthritic subjects attained more nearly normal areas. These data show that the rheumatic patient suffers from a disorder of the physicochemical pattern of his blood as well as from a pathologic process in his joints.

While these deviations apparently bear a general relation to the degree of systemic involvement, they should not be regarded as pathognomonic for arthritic diseases. Some evidence is presented indicating the probability that a major factor determining the magnitude of the films relates to variations in the concentration of proteins in the specimens studied. Full interpretation of the observed differences requires more data than are now available, but there are reasonable grounds for the speculative view that certain physiologic consequences must follow upon a reduction in the "film-forming stuff" in body fluids. An evaluation of the role of film-forming capacity of blood fluids in physiologic processes and the modifications in pathologic states invites further inquiry.

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VITAMIN A AND THE DETOXICATION OF MONOBROMOBENZENE*

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THE vital relationship of cystine to insulin, glutathione and other functionally important sulfur compounds, led us to experiment with monobromobenzene, which according to White and Jackson[1] and Stekol,[2] is specifically detoxicated by the sulfur containing amino acids, removing them from the normal nutritional processes.

In our experiments,[3] animals were fed quantities of monobromobenzene far in excess of the amount that could have been detoxicated if all of the sulfur compounds of the diet were utilized for that purpose, and led us to suspect other modes of detoxication.

Goerner[4] working in this laboratory has shown that certain carcinogenic hydrocarbons cause a rapid depletion of the liver vitamin A. This suggests that this vitamin may play a role in the detoxication of aromatic hydrocarbons and related compounds.

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In this work we have attempted to show a relationship of the vitamin A content of liver tissue to the detoxication of monobromobenzene.

EXPERIMENTAL

The experimental work is divided into three phases. The first phase is a study of the effect of injected monobromobenzene on vitamin A deficient rats; the second, the feeding of monobromobenzene to normal rats and determining its effect upon the liver vitamin A level; and the third, the measuring of the effect of subcutaneous injection of monobromobenzene at various levels over various time intervals upon the liver vitamin A level.

Experiment No. 1.—A small series[15] of white rats that had been depleted of vitamin A by the U.S.P. method and an equal number of normal control animals were given subcutaneous injections of 100 mg. of monobromobenzene in the form of a 10 per cent solution in corn oil.

All of the rats in the vitamin A depleted group died within 48 hours after injection, while the normal animals showed no harmful effects.

Experiment No. 2.—A group of 20 adult white rats, designated A, were placed upon a normal laboratory feed (0.54 per cent S) that had been supplemented with 2 per cent of monobromobenzene. The monobromobenzene was incorporated by mixing it with twice its weight of corn oil before adding it to the stock diet. The prepared diet was stored in airtight containers. An equal number of control rats, designated B, were maintained on the unsupplemented stock diet. At intervals varying from three weeks to three months a few animals from each group were killed and their livers assayed for vitamin A content. The vitamin A was estimated by extraction according to the method of Moore[5] and the vitamin determined by the Andersen and Levine[6] modification of the method of Rosenthal and Erdelyi,[7] using a biologically standardized vitamin A concentrate as a standard for this colorimetric procedure.

The results of this experiment are tabulated in Table I.

Experiment No. 3.—Four series of adult white rats were placed upon a normal complete diet and given subcutaneous injections of monobromobenzene dissolved in corn oil (50 mg. per c.c. of oil).

Series A received 50 mg., series B 100 mg., series C 150 mg., of monobromobenzene, and series D 2 c.c. of corn oil without monobromobenzene.

The injections of monobromobenzene were made three times each week for a four-week period. At the end of this time the animals in all four series were killed and their livers assayed for vitamin A content (Table II).

DISCUSSION OF RESULTS

The first experiment shows that animals depleted of vitamin A have an extremely low capacity to withstand the toxic effect of monobromobenzene. This poor resistance cannot be entirely related to the absence of vitamin A, since such animals are in poor physical condition.

In the second experiment the lower vitamin content of the animals receiving the monobromobenzene is definite evidence of a harmful effect of this agent.

TABLE I

GROUP A			GROUP B		
RAT NO.	NUMBER OF DAYS ON DIET	TOTAL VITAMIN A CONTENT OF LIVER	RAT NO.	NUMBER OF DAYS ON DIET	TOTAL VITAMIN A CONTENT OF LIVER
1.	21	1130 units	21.	21	990 units
2.	21	650 units	22.	21	700 units
3.	28	1700 units	23.	28	1150 units
4.	28	1050 units	24.	28	1100 units
5.	35	460 units	25.	35	1160 units
6.	35	650 units	26.	35	870 units
7.	42	900 units	27.	42	730 units
8.	42	150 units	28.	42	650 units
9.	50	870 units	29.	50	1010 units
10.	50	800 units	30.	50	950 units
11.	62	55 units	31.	62	750 units
12.	62	620 units	32.	62	1170 units
13.	71	1040 units	33.	71	650 units
14.	71	610 units	34.	71	800 units
15.	78	630 units	35.	78	850 units
16.	78	220 units	36.	78	850 units
17.	85	380 units	37.	85	940 units
18.	85	140 units	38.	85	620 units
19.	92	400 units	39.	92	880 units
20.	92	350 units	40.	92	1030 units

Group A received 2 per cent monobromobenzene in diet.
Group A lost 0.11 grams per rat per day while on diet.
Group B lost 0.06 grams per rat per day on the normal diet.

TABLE II

SERIES A		SERIES B		SERIES C		SERIES D	
RAT NO.	TOTAL LIVER VITAMIN A	RAT NO.	TOTAL LIVER VITAMIN A	RAT NO.	TOTAL LIVER VITAMIN A	RAT NO.	TOTAL LIVER VITAMIN A
51.	760 units	61.	440 units	71.	800 units	81.	880 units
52.	830 units	62.	760 units	72.	1260 units	82.	750 units
53.	270 units	63.	910 units	73.	850 units	83.	910 units
54.	680 units	64.	900 units	74.	1020 units	84.	390 units
55.	1100 units	65.	650 units	75.	800 units	85.	1100 units
56.	940 units	66.	870 units	76.	590 units	86.	850 units

Series A received 50 mg. monobromobenzene three times each week.
Series B received 100 mg. monobromobenzene three times each week.
Series C received 150 mg. monobromobenzene three times each week.
Series D received no monobromobenzene.

It cannot be assumed, however, that the vitamin decrease is directly due to its action in detoxifying the monobromobenzene, since there is the possibility that the monobromobenzene interferes with the absorption of the vitamin of the diet. The fact that only minor weight changes occurred during the experimental period proves that any interference with the absorption processes is not extensive.

The injection procedure of the third experiment did not cause a noticeable change in the liver vitamin A level and proves the lower vitamin A levels obtained in the second experiment are not due to a detoxication process but are possibly associated with a failure to absorb the vitamin from the intestinal tract in the presence of the monobromobenzene.

SUMMARY

Vitamin A does not appear to be associated with the detoxication of monobromobenzene, although vitamin A deficient animals have a lowered resistance to this toxic agent.

There is some evidence that monobromobenzene interferes with the absorption of vitamin A.

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SYRINGADENITIS SUPPURATIVA TROPICALIS (A COMPLICATION OF LICHEN TROPICUS)*

Histologic Appearance and Etiologic Considerations Particularly as to a Possible Relationship of Ascorbic Acid and Carbohydrate Metabolism

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THE observation has been made by competent observers that few persons in the tropics escape being affected by lichen tropicus (prickly heat). Those without prior history of attack are generally free or are mildly affected during their stay in hot climates, whereas those previously affected are usually subject to subsequent and increasingly severe attacks of this very annoying disease. Only a limited number of patients with prickly heat develop complications in the form of multiple furuncle-like lesions. Since only a few references have been made in the literature to this complication, it was considered important to present the subject not only from a general climatic-pathologic but also from a dermatologic point of view.

Without going into a detailed discussion, it should be noted that no acceptable explanation has been yet offered regarding the cause of lichen tropicus. Unna[1] considered it an inflammatory disease of the sebaceous glands but also was

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aware of an edematous swelling and obstruction of the epidermal cells due to disturbances of sweat secretion. Pollitzer[2] was partly in agreement with Unna[1] and he emphasized the obstruction of the flow of the sweat with subsequent sweat imbibition and swelling of the epidermal cells. Kayser[3] stressed the irritating effect of urea and sodium chloride of the sweat, whereas Ziemann[4] finds an explanation in high atmospheric humidity. Acton[5] speaks of an inflammation of the sweat glands caused by staphylococci and Smith[6] considers *Monilia* as the etiologic agent. Very little investigative work has been done to shed light upon the complications of the subsequent suppurative involvement of the sweat glands. Most textbooks make few references to it and many do not even mention the existence of these after-effects of prickly heat. Castellani[7] stated that "occasionally the vesicles may become purulent and crops of boils may develop." Manson-Bahr[8] agreed that prickly heat is a common though an indirect cause of boils; for the "breaches of surface following on the scratching it induces afford many opportunities for invasion of the micro-organisms of furuncular disease." Plehn[9] (in Mense's handbook) refers to the lack of cleanliness and scratching which may be blamed for extensive lesions and deep suppurations. In recent literature Acton and Smith[5, 6] make brief mention of its occurrence in India and Nigeria, respectively. G. Pernet's[10] histopathological interpretation of Radcliffe-Crockers case of acne agminata should, however, be mentioned, this being the first observation of similar nature. "The primary change appeared to occur about the sweat coils which were more or less disorganized by an inflammatory leucocytic infiltration. In parts, a hair follicle had also become involved and necrotic changes had taken place in the central parts of the infiltration. There is also a perivascular infiltration. The origin about the sweat gland agreed with the clinical evolution of the individual lesions, which commenced in the deeper parts and gradually came up to the surface with suppuration. The disease had started somewhat suddenly, about the end of August, 1901, while the patient was at the seaside for his holiday. No plasma cells were found by Pappenheim's method; few sections were stained for tubercle bacilli with negative results."

CLINICAL APPEARANCE OF LICHEN TROPICUS

Lichen tropicus (prickly heat) makes its appearance when the temperature is around or above 90°, in regions with high atmospheric moisture. The onset is characterized by a severely itching disseminated, follicular, periporitic vesicular eruption which the naked eye would recognize as a small follicular macule. The most favored regions are the covered parts of the body, especially the flexoral folds where often the eruption first appears. Only exceptionally is the face more severely involved than the rest of the body. The tiny vesicles soon become turbid, rupture, and occasionally an eczema-like plaque appears which adds to the already existing irritation. With the change to cool weather or when proper treatment is given, the eruption subsides. This is followed by a fine desquamation.

1 *Complication of Lichen Tropicus.*—As already pointed out, only a limited number of people show severe involvement of the face and these are the persons who are frequently subject to a peculiar complication. Though itching may

be absent on the face or be considerably attenuated, there appears instead a growing sensation of tension over the face (rarely over the buttocks or the submammillary regions) which is followed by the appearance of numerous painful split-pea-sized and larger superficial and deep nodules (Fig. 1). These lesions at the onset are covered by shiny red skin which, after several days, may either develop a purulent top with subsequent suppuration of deeper portion of the lesions, or the lesions may disappear after four to eight weeks by absorption. Many weeks after the disappearance of the lesions there still remains a visible trace in the form of a distinct hyperpigmentation. Scarring occurs occasionally. The course of the disease is limited to from one to two and one-half months, during which time patients are incapacitated, not only on account of the unsightly appearance but at times also on account of pain and constitutional symptoms. Regional lymph nodes are slightly enlarged but never suppurative. This complication has been named syringadenitis suppurativa tropicalis.

Fig. 1.—Profuse outbreak of nodular lesions with limited involvement of the upper and lower eyelids. Note the complete freedom of the center of the upper lip and of the chin.

Fig. 2.—Lesions are numerous on the bridge of the nose and forehead, but the periocular or perioral regions are hardly affected.

Occurrence of the Complication.—Of twenty cases personally investigated, the face alone was affected in sixteen. The forehead and the bridge of the nose showed the severest involvement, whereas the periocular region, the upper lip, especially the center (philtrum) and the lower lip and chin were free (Fig. 2). In two cases a few nodules developed on the buttocks of two men and in two women a few lesions were observed under the breasts. In not a single instance were the axillae, the palms or the soles involved.

HISTOPATHOLOGY OF THE COMPLICATION

On account of the site of the lesions only a very limited number of women gave their consent for biopsy. Microscopic examination of the 20 cases were therefore made in only 8 instances. In four cases serial sections were made.

Epidermis.—In general the epidermis shows the following characteristic changes. In the majority, a distinct enlargement of the sweat and follicular orifices is observed. In an equal number of cases a subcorneal cavity is filled with detached, poorly staining cells, serofibrin and in later stages with leucocytes and organisms. The pustules are intraepidermal (producing splitting of the layers), the floor being composed of an edematous rete malpighi and the roof of lightly stained edematous prickle cells. The contents showed necrotic cells, leucocytes, and cocci. The surrounding area is distinctly edematous. These changes correspond to the position of sweat glands and in sections one can frequently trace an inflammatory focus above a sweat gland.

Corium.—The corium is the seat of major changes (Fig. 3). The papillary capillaries are dilated and surrounded by leucocytes and lymphocytes and occa-

Fig. 3.—Low power photomicrograph showing: dilated pilosebaceous orifices; cellular breakdown above a sweat gland, with partial involvement of the coils.

sional plasma cells. The sweat ducts are also distinctly dilated and invaded by leucocytes and poorly staining cocci. This change increases with the beginning of the sweat duct. Not a single duct shows a cellular breakdown. The area above the sweat gland is generally the seat of cellular disintegration where small cavities are formed filled with necrotic cellular debris, leucocytes, and cocci. The coils are frequently also involved in the disintegrating process; there is no conclusive evidence as to whether the primary seat is the glandular parenchyma or whether the infection is transmitted through the duct or from the surrounding area. But it seems very suggestive that the infection travels partly via the sweat duct. Rarely, perifollicular abscesses involving the sebaceous glands are noticed. Most of the sections, however, reveal a tendency for abscess formation to develop above the sweat glands and only in rare instances is the localization a haphazard one. The cutaneous lymphatic spaces are dilated and infiltrated with inflammatory cells. At times one gains the impression as if the process would spread by the lymphatics.

CLINICAL NOTES

Our investigation of syringadenitis suppurativa tropicalis was carried out during the months of June, July, and August in 1939, 1940, and 1941 and is based on the observation of twenty Chinese patients (eighteen females, two males). The youngest was 17 years of age and the oldest 26. While this report deals only with Chinese, it should be noted that a similar complication is occasionally met with in other races in the same locality, whose occupation demands an outdoor life (at times also after long games of tennis, golf, etc.). A striking feature in our cases was the prevalence of the disease on the face and the predominating number (90 per cent) of young women (farmer, gardener class). Lack of cleanliness of the face can be safely discarded as a possible cause because the Chinese indulge in frequent wiping of their faces with towels dipped in boiling water. It could also be considered that scratching could be a contributory factor for infection. The fact, however, is the least irritated part of the skin and is hardly ever scratched. These possible exogenous etiologic factors were therefore partially eliminated and our attention was drawn to certain climatic and metabolic considerations. The staple food of the class of Chinese under our investigation consists of vegetables and polished rice. The intake of vitamin C during the summer is adequate and a nutritional insufficiency seemed unlikely. For the sake of comparison it should be mentioned that on account of the lack of green vegetables, the ascorbic acid level in the blood serum of the Chinese (in Peking) is considerably lower during winter. This, according to T. F. Yu,[11] is not due to racial difference because the serum ascorbic acid level goes up immediately as soon as ascorbic acid is added to the diet.

In tropical Java, de Haas[12] investigated the vitamin C serum level of Chinese and found that 0.86 mg. per cent was the average for 42 healthy persons. Our (unpublished) observations in healthy individuals during the summer months gave substantially the same results. We expected therefore that the excessive perspiration may cause an appreciable loss of vitamin C. This alteration may render the depleted skin susceptible to infection. Furthermore, a disturbed carbohydrate metabolism was expected, basing our supposition on the fact that an increased blood or tissue sugar content is favorable for the development of pyogenic infections of the skin. Our investigation included (1) blood serum vitamin C determination (according to Pijoan and Klemperer[13]), (2) blood sugar determinations (according to Hagedorn and Jensen).[14]

Serum Vitamin C Determinations.—Among the 20 patients investigated, the lowest level was 0.05 mg. per cent and the highest 0.78 mg. per cent. The average was 0.15 per cent. The daily administration of 200 to 300 mg. of ascorbic acid (Redoxon "Roche") increased the blood serum vitamin C level only moderately (10-20 per cent) during the period of excessive perspiration, whereas there was a definite rise with the onset of cooler weather and subsiding perspiration. This gives an indication of a possible partial pathomechanism for the vitamin C depletion and may also explain to a certain extent our difficulty in obtaining a saturation level of vitamin C during the period of excessive perspiration.

BLOOD SUGAR TOLERANCE TEST

Among the 20 cases investigated, the lowest fasting glucose level was 40 mg. per cent and the highest 70.5 mg. per cent making an average of 50.5 mg. per cent. After the intake of 1.75 Gm. of glucose per kg. of body weight, the blood sugar concentration showed a rapid rise (30-50 per cent) in the first fifteen minutes. After forty-five to sixty minutes, there was a drop of 15-20 per cent as compared with the initial rise. After two hours the level reached its highest peak with an increase of 40-50 per cent as compared with the fasting blood sugar level. After three hours, a 40-50 per cent increase was still present. This pattern of blood sugar tolerance has been observed in 80 per cent of the cases that were studied. A typical example is Chang Chen Chi, 17-year-old girl (O.P.D. No. 137149). (Chart 1.)

Glucose was not detected in a single specimen of urine.

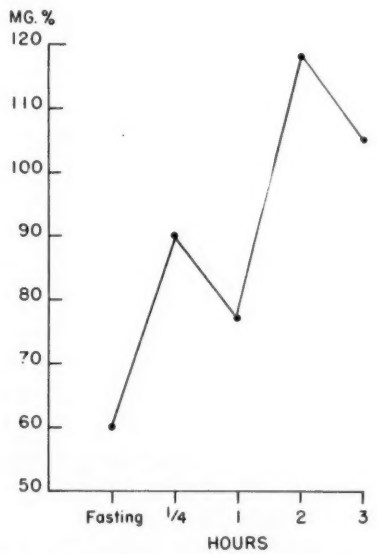

Chart 1.—Typical glucose tolerance curve.

INTERPRETATION OF DATA

In order to correlate the low serum ascorbic acid, with the low fasting blood sugar level, and the delayed glucose excretion, it may be of interest to recall the many investigations dealing with the relationship and with the effect of ascorbic acid on carbohydrate metabolism. It may be stated at once that there is a great variation in the reported results which is probably due to the different methods in use. While a number of investigators found that the administration of ascorbic acid to diabetics lowered the blood sugar; others such as Asinelli,[15] Mosonyi and Aszodi,[16] Stoïcesco and Gingold[17] and Tarsitano and Spena[18] found that the administration of ascorbic acid increased the level of blood sugar. Wille[19] found that vitamin C medication increased the blood sugar level especially under hypoglycaemic conditions. Stepp[20] and Schroder[21] and Altenburger[22] investigated the glycogen mobilizing effect of adrenalin. They found

a higher blood sugar peak after combined ascorbic acid medication in comparison with adrenalin alone. Kreitmair[23] confirmed these findings with his experiments on cats. Hamne[24] gave ample proof in his excellent monograph of the relationship of carbohydrates and vitamin C metabolism. According to his very carefully executed studies, the glucose tolerance curve showed an abnormal course in hypovitaminosis C. Not only was an excessive rise of blood sugar observed, but the post alimentary insulin secretion was provoked much later in comparison with cases with a normal vitamin C standard. It should be noted that animals kept on a low vitamin C diet develop a compensatory hypertrophy of the islands of Langerhans. (Rondoni and Montagnani,[25] Monasterio,[26] Borghi[27] and De Otto.[28]) These findings are parallel with the conclusions reached by Dessy and Doneddu[29] who found that both in normal persons and in diabetic patients intravenously administered ascorbic acid was recovered in greatly reduced amounts when a simultaneous intramuscular injection of insulin was given.

There is little doubt that the close interrelationship between the carbohydrate metabolism and vitamin C is firmly established. Apparently vitamin C plays in the carbohydrate metabolism the role of a bio-regulator. In other words vitamin C enters into the regulation of the blood glucose level in an important way in that it increases this level in hypoglycemia and decreases it in hyperglycemia. This interpretation would also offer a reasonable explanation for our findings.

Not only is the low fasting blood sugar level compatible and explicable with the low vitamin C blood serum level but the abnormal glucose tolerance test is a further characteristic in cases of vitamin C deficiencies. The high blood sugar curve after 2 hours indicates a lowered tolerance for carbohydrates and the persistent pathoglycemic peak after 3 hours serves as an added confirmation to the foregoing statement. The unexpected reduction of the blood sugar level after the initial rise is due to the effect of the low vitamin C level which stimulates insulin secretion (insulin effort phenomenon). This sudden effort apparently hinders the subsequent insulin secretion, which supposition is well based on the renewed rise of the blood sugar level which almost bears a certain resemblance to the blood sugar curves seen in diabetic patients. How far the adrenals are involved in this mechanism on account of the low vitamin C level, or are altered in their function on account of the enormous loss of sodium chloride through perspiration should be made a subject of further studies. It is, however, noteworthy to mention that McQuarrie[30] observed a more efficient carbohydrate utilization with a high sodium chloride medication and MacLean[31] gave evidence that the simultaneous sodium chloride medication in diabetic patients has reduced the insulin requirements.

CLIMATIC FACTORS

All of our patients lived largely an outdoor life whereby they were exposed to the effects of the sun during June, July, and August combined with the excessive atmospheric humidity and high temperature. No studies regarding climatic physiology of the skin has been carried out by us, but it is of interest to relate the experiences of Marchionini and Tor.[32] These authors have found in Anatolia (Turkey) during the summer months an enormous

increase of pyodermic infections of the face especially in individuals from ten to twenty years, females predominating. The authors offer proof that not only experimentally after ultraviolet ray exposure but also in individuals exposed to the sun, (1) the pH of the skin is shifted toward the alkaline side from 5.24 to 7.28); (2) in the dialysate of the skin the sugar, calcium and catalase increases and the amylase decreases.

Marchionini's[32] skin glucose findings, have not been correlated with blood sugar examinations. It should, however, be mentioned that low blood sugar figures do not exclude higher skin sugar findings in the same individual as the investigations of Urbach[33] have demonstrated. Whereas H. Laurens[34] states that "irradiation produces a lowered blood sugar and increases sugar tolerance." While no confirmation of Marchionini's findings has yet been reported, it seems a fair and logical working basis to explain the predisposition of the face for pyogenic infection of our cases. The shifting of the pH of the skin towards the alkaline side would certainly render the skin surface favorable for staphylococcus infection more especially in a skin in which the sugar concentration has been increased. The increase of catalase could indicate an increased oxidation-reduction which in our cases may explain the reduced vitamin C level of the blood. This depletion could furthermore be interpreted as a partial loss of excessive perspiration knowing that the sweat contains approximately 0.08-0.5 mg. per 100 c.c. and partly also in the later stages on account of the inflammatory purulent process knowing that the presence of oxidative substances in staphylococci especially demands an increased vitamin C metabolism (Harris).[35]

BACTERIOLOGIC FINDINGS

Cultures were made on 2 per cent glucose-agar from the superficial follicular vesicles, and also from the deeper lesions. In not a single case could we obtain *Monilia,* but in 19 cases *Staphylococcus aureus* was obtained in pure culture, whereas in 2 cases a scanty growth of *Staphylococcus albus* and *Staphylococcus citreus* were found.

DIFFERENTIAL DIAGNOSIS

Without previous knowledge of the clinical features, the first impression is suggestive of an iodide or bromide eruption. These can usually be ruled out because of a negative history of halogen ingestion. Against pustular syphilide are the acuteness of the lesions and the negative serologic finding. Furunculosis can be ruled out because of the absence of necrotic cores and because the localization is not strictly follicular. The symmetry is not a characteristic feature of either furunculosis or impetigo. Pyogenic infections following mosquito bites are frequent in China but favor more often the lower extremities. In all such instances, we see more or less impetiginous-crusted lesions developing at the site of scratching. From bacterids the differential diagnosis is usually not difficult because of the absence of an infectious focus and because of the unusual localization. From Harara (Heat disease of the face in Palestine) described by Dosztrovsky[36] this affection can be also differentiated because of the absence of phlebotomus in the Shanghai region, the

stinging of which is the cause of the disease. In its clinical appearance, syringadenitis suppurativa tropicalis is similar to varus nodulosus (Brook),[37] but the possibility of confusion is eliminated by the fact that varus nodulosus has a chronic course and long duration and also because of the absence of vesiculation and suppuration. Acne agminata (disseminated follicular lupus of Tilbury and Fox, acnitis of Barthelemy, hydradenitis destruens suppurativa of Politzer) should be mentioned not only because of the localization on the face, but also because of the long controversy whether the sweat-glands are the sites of primary involvement. The coppery colored lesions, their sluggish onset, the indolent nature of the disorder, the tuberculoid histologic changes are features not present in the disease under discussion in this paper.

DISCUSSION

Inflammatory and suppurative conditions affecting apocrine sweat glands are well known (Hydradenitis suppurativa). The only disease entity which concerns inflammation of the eccrine sweat glands, are the multiple abscesses of infants. Though the age incidence is entirely different, the clinical picture is similar with pea-to-hazelnut size, reddish, disseminated, hemispheric swellings which become fluctuating and discharge a greenish pus. The causative agent is *Staphylococcus albus* and the histopathology has a good many features in common with syringadenitis suppurativa tropicalis. In multiple abscesses of infants the onset is a periporitic pustule which penetrates into the deeper strata of the cutis and finally reaches the subcutis in the region of the sweat glands which at times are also involved in the process though often remain intact. In the disease under discussion in this paper it is also rather suggestive, but not quite obvious, that the process is a direct continuation of the invading infection through the sweat ducts. It is, however, also analogous that the infection may be carried from the vicinity to the sweat glands.

Pyogenic infections of the eccrine sweat glands of adults are not known in the temperate zone, and only certain climatic conditions and metabolic changes such as occurred in our patients would explain such an eventuality. The dilated sweat pores in prickly heat are not only a natural port of entrance for pyogenic cocci, but the irritation due to profuse perspiration renders the sudoriparous apparatus a locus minoris resistentiae. To this we have to add the diminished bacteriostatic power of the greatly diluted sweat and the possible changes brought about by the action of sunlight (increased sugar of the skin, etc.). While with large doses of vitamin C (900 to 1000 mg. daily) an appreciable improvement was noticed, smaller doses, 2 to 300 mg. were only effective when the hot weather subsided and perspiration decreased. The serum vitamin C and blood sugar level could only be increased with difficulty during the hot season, but both improved simultaneously with the onset of the cooler weather. These observations lead us to conclude that through the excessive loss of vitamin C the carbohydrate metabolism was disturbed. Though the fasting blood sugar level was rather low, the glucose tolerance test showed not only an abnormal high postcibal range, but also a delayed excretion of the sugar. The possibility of an increased glucose content of the skin, more

especially of the exposed parts, must be considered. The findings of Marchionini and Tor,[32] in respect to cases similar to our own, appear fairly reasonable because in our cases the facial localization was also a predominant feature and the covered parts seldom revealed lesions of a similar nature, though the frequent scratching, particularly of the covered regions affected by prickly heat, would greatly favor an infection. How far, besides the disturbed carbohydrate-vitamin C equilibrium, other colloidal chemical changes of the sweat gland parenchyma are involved is a matter of conjecture and beyond the scope of the present report.

TREATMENT

Treatment consisted of conservative measures, though in a few cases small incisions were made. Otherwise the local treatment depended chiefly on the stage of the disease. In case of pain, compresses with a soothing lotion was applied. In cases where the lesions were nearing breakdown, application of the following ointment was found effective:

Ichthyol	10.0 Gm.
Hydrarg. oxyd. rubr.	0.2 Gm.
Resorcin	0.4 Gm.
Vaseline	ad 20.0 Gm.

Internal medication included ascorbic acid, 200-1000 mg. daily. Neither the sulfonamide drugs (sulfathiazole or sulfapyridine) nor toxoid gave us any encouraging results.

CONCLUSIONS

1. An inflammatory and suppurative disorder of the eccrine sweat glands occurring in Chinese patients has been described under the name syringadenitis suppurativa tropicalis.

2. The disease is thought to be a complication of lichen tropicus (prickly heat).

3. The causative agents of the disease are assumed but not proved to be the *Staphylococci aureus* and *albus*.

4. Evidence is advanced that the inflammatory involvement and infection of the sweat glands is furthered by an excessive loss of vitamin C and altered carbohydrate metabolism.

5. Treatment consists in the use of appropriate topical applications and vitamin C internally.

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THE DIFFUSION OF DYES IN ETHYLENE GLYCOL GELS

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INTRODUCTION

SCHULEMANN[13] has pointed out the relation between the diffusion of a dye and its usefulness as a tissue stain. Regardless of whether the mechanism of staining is explained by the formation of chemical compounds between the dye and the tissue[14] or by the adsorption of the dye by the tissue,[9] the rate of penetration of the dye is a factor which must be considered in the choice of a dye as a stain. Stiles[15] has shown that conditions in dilute gels duplicate closely those present in tissues. Fautrez and Lison[4] have emphasized that while diffusion in a gel is a complicated process and should not be expected to give true values of the dimensions of the diffusing particle, nevertheless diffusion in tissues and in gels are similar processes and are controlled by the same factors. Accordingly, the diffusion of dyes in gels should throw some light on the usefulness of dyes as stains.

The diffusion of certain acid and basic dyes in ethylene glycol gels has been measured. Among the acid dyes investigated were: orange II, merbromin, metanil yellow, congo red, and pontamine blue BBF. Chrysoidine Y was chosen as a representative of the basic dyes. Diffusion took place in 5 per cent gelatin gels of 80 per cent buffered ethylene glycol. Ethylene glycol was chosen as the solvent, because Geschickter[7] has used it successfully in this capacity in staining tissue cultures. Its antiseptic properties are advantageous in preventing the growth of bacteria in the gel over a long period of time.

METHOD

As the buffer salts (sodium hydroxide and potassium acid phosphate) required to make gels of the desired hydrogen ion concentration are insufficiently soluble in ethylene glycol, some water had to be added. For uniformity, all solutions were made to contain 80 per cent of the glycol, the rest being water and buffer salts as required. This solution was used in the preparation of 5 per cent gelatin gels. The hydrogen ion concentration of sample solutions was measured by Dr. W. C. Harden of Hynson-Wescott-Dunning, Baltimore, Md., with the quinhydrone electrode and colorimetric standards prepared from these solutions were used as checks. Solutions of pH 5.5 and pH 7.65 were used.

The unsolidified gel was divided into two portions. One portion was poured into a 16 mm. test tube until the tube was approximately half full and allowed to solidify in the thermostat at 0° C. To the other portion was added

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sufficient dye to make a solution M/200 in actual dye content. The gel containing the dye was poured over the solidified clear gel of the same pH and placed in the thermostat. The dye was therefore diffusing into a medium identical in all respects, except for the presence of the dye, with that in which it was dissolved.

In the measurement of the diffusion, a photometer was used to compare the amount of light transmitted through a neutral glass filter and through the gel containing the dye solution. Two glass filters were used corresponding to different concentrations of dye, c_1 and c_2, with c_1 representing a larger concentration of dye than c_2. Daily measurements were made of the distances (d_1 and d_2) from the initial concentration of the dye, M/200, to the concentrations c_1 and c_2 measured by the photometer. In principle, this method is similar to that used by Furth[6] and by Fautrez and Lison[3] who measured the distances the dye diffused to reach a concentration which was a definite fraction of the initial concentration. Their experimental arrangements differ from those described in this paper in that they used microtechnique and measured diffusion in the solvent alone rather than in a gel.

The boundary between colored and colorless gel was also measured. Here the setting was not a photometric one; the line of demarcation between colored and clear gel as intensified by the optical instrument was measured. The symbol d_3 represents the distance of diffusion of the dye from its initial concentration, M/200, to a concentration so small that to visual observation it appears to be zero.

The laboratory measurements of two or more different samples of the same dye were plotted, using as ordinates the distances in mm. and as abscissae the time in days. A smooth curve was drawn between the points and the distances read from this curve. Fig. 1, in which the circles and triangles represent readings from two samples, illustrates the procedure followed and indicates the amount of error involved in the measurement.

RESULTS

In Table I are assembled the distances in mm. which the dyes diffused at the end of 20 days.

TABLE I

DISTANCES OF DIFFUSION IN MM. AFTER 20 DAYS

DYE	pH 5.5			pH 7.65		
	d_1	d_2	d_3	d_1	d_2	d_3
Congo Red—com.	9.2	10.6	12.85	9.45	11.0	12.4
Congo Red—purified	9.0	10.4	12.9	9.5	10.6	12.3
Pontamine Blue BBF	10.05	11.1	12.1	9.5	10.6	11.6
Orange II	13.4	14.6	16.85	14.0	15.6	17.55
Metanil Yellow	13.6	14.9	16.8	14.05	15.85	17.6
Chrysoidine Y	14.75	16.4	18.6	17.4	18.45	19.45
Mercurochrome				13.6	14.4	15.45

Conn[2] has shown that impurities present in the dye may affect its usefulness as a stain. As some of the dyes were technical samples and others carefully purified, it was important to discover whether the presence of impurities commonly found in technical samples would influence diffusion when

the actual dye content of the two samples was the same. The differences in the diffusion of a commercial sample of Congo Red and of a purified sample were found to be negligible, usually 0.2-0.3 mm. or less. This statement is based on a daily comparison of the diffusion distances of duplicate samples of the two dyes over a period of 30 days. Probably the buffer salts in the solution make the effect of additional salts present in the commercial sample negligible. These results are in agreement with the work of Valko.[17]

Table I gives the results of measurements at the two pH values investigated—one of them, pH 5.5, being very near the isoelectric point of gelatin; the other, on the alkaline side. There is little regularity in the slight differences in distance at the two pH values for the dyes with higher molecular weight; the dyes of lower molecular weight, which may be considered in true solution, show a slight but regular tendency towards more rapid diffusion in the solutions of higher pH. Mommsen[10] and Pischinger[12] in studying the effect

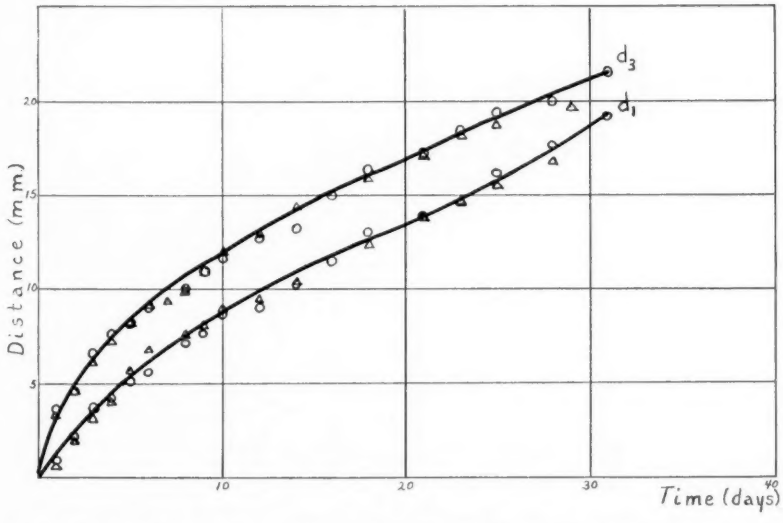

Fig. 1.—Metanil Yellow pH 5.5. Circles and triangles represent measurements on different samples.

of changing pH on the diffusion of dyes in gelatin gels have found that acid dyes diffuse more readily in less acid media and basic dyes more readily in less basic solutions. Their experiments cover a wider range of pH, especially on the acid side; our experiments fall within the pH range in which Pischinger found very slight changes in the diffusion distances.

From Stefan's development of Fick's law, the square of the amount of substance diffusing divided by the time should have a constant value if the initial concentration of the diffusing substance and the cross section in which diffusion is taking place remain unchanged. Stefan found that the same relationship should be true if the amount of substance is replaced by the distance of diffusion to a definite concentration, thus giving a constant value proportional to the diffusion constant. These relations were tested experimentally and found true, at least as a first approximation.[18]

Values of $\frac{d^2}{t}$ were calculated from the three measurements described above. It is evident from Fig. 2, in which values of $\frac{d^2}{t}$ are plotted as ordinates against corresponding values of t as abscissae, that in the case of the first two measurements, d_1 and d_2, constants were not obtained, but that with the third measurement, representing diffusion from a solution M/200 to an in-

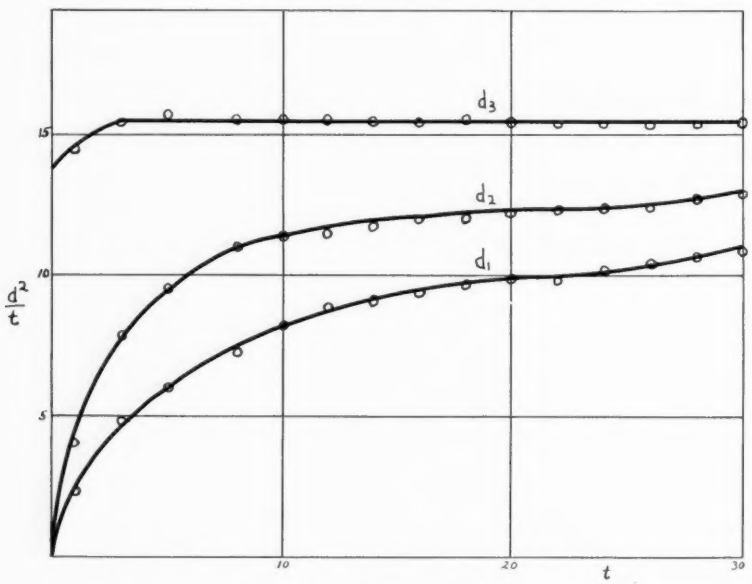

Fig. 2.—Orange II pH 7.65.

finitely dilute concentration of dye, a constant resulted. Further inspection shows that in the first two measurements, values which should be constant increase with increase in time. This suggests a logarithmic curve. Figs. 3 and 4 represent logarithmic curves for diffusion distances of typical dyes. Logarithms of the distances diffused were plotted as ordinates with logarithms of the corresponding times as abscissae. Straight lines were obtained with the first two measurements, d_1 and d_2, as well as with the third measurement, d_3. The slope of the line for d_3 is 2, giving constant values for $\frac{d^2}{t}$, but the slope of the lines for d_1 and d_2 is some fractional value between 1 and 2. When values of $\frac{d^n}{t}$ are calculated for the distances represented by d_1 and d_2, with n representing the slope as determined from the logarithmic curves, constants are obtained. In Table II, values of $\frac{d^2}{t}$ are compared with values of $\frac{d^n}{t}$ for a typical case. In Table III, values of the slope, n, and of the constant, k, from the expression, $k = \frac{d^n}{t}$, are assembled for the various dyes.

DISCUSSION

Many of the logarithmic plots show a different slope at the beginning of the process from that followed during the later stages (see Figs. 3 and 4).

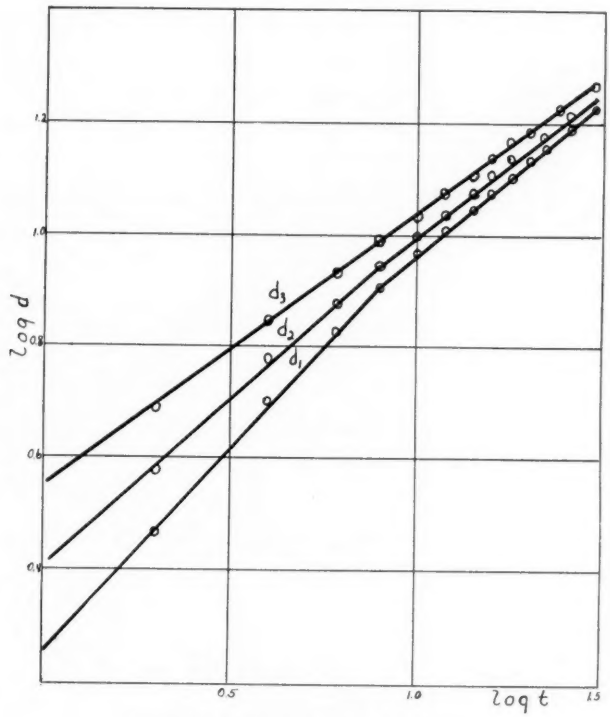

Fig. 3.—Mercurochrome pH 7.65.

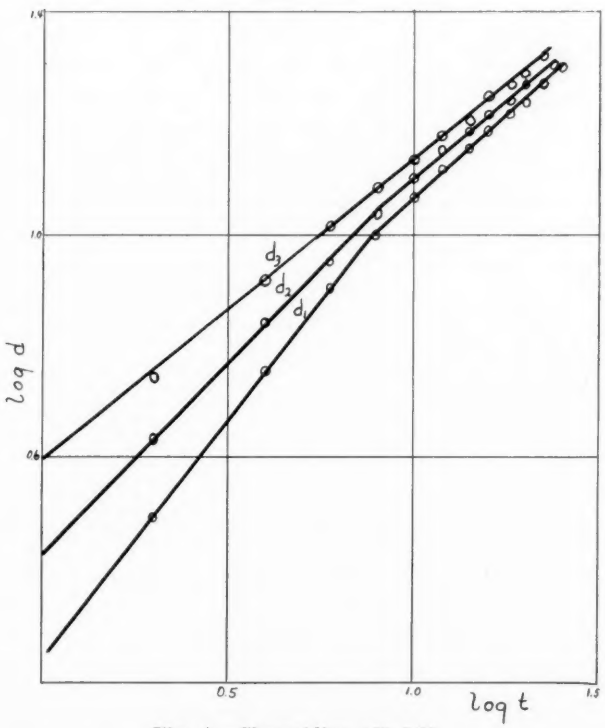

Fig. 4.—Chrysoidine pH 7.65.

This means that the advance to a definite concentration is slower and that n has a lower value in the initial stages of penetration. Similar results have been observed by Stiles[15] and by Bigwood[1] in studies of the penetration of electrolytes and of dyes. Change in structure of the gel with ageing may account for the increase in diffusion. In such cases the values of n in Table III are taken after the change in slope which occurs when diffusion has taken place for 8-10 days.

TABLE II

CALCULATION OF DIFFUSION CONSTANTS

CONGO RED, pH 7.65

n is a constant representing the slope of the logarithmic curves

t	$\frac{d_1^2}{t}$	$\frac{d_1^n}{t}$	$\frac{d_2^2}{t}$	$\frac{d_2^n}{t}$	$\frac{d_3^2}{t}$
3	1.61	1.04	3.41	2.18	7.05
6	2.66	1.23	4.51	2.38	8.05
9	3.24	1.26	4.98	2.39	7.93
12	3.63	1.26	5.33	2.39	7.68
15	3.95	1.26	5.28	2.27	7.63
18	4.30	1.27	5.44	2.24	7.61
21	4.57	1.27	5.66	2.24	7.56
24	4.77	1.26	5.90	2.26	7.71
27	4.98	1.26	5.97	2.23	7.78
30	5.21	1.26	6.16	2.25	7.80
33	5.28	1.24	6.28	2.24	7.80
Average		1.26		2.28	7.69

TABLE III

SUMMARY OF DIFFUSION CONSTANTS OF DYES

FROM THE EQUATION $k = \frac{d^n}{t}$

DYE	d_1 n_1	d_1 k_1	d_2 n_2	d_2 k_2	d_3 n_3	d_3 k_3
			pH 5.5			
Congo Red	1.27	0.80	1.446	1.47	2	8.19
Congo Red—com.	1.23	0.73	1.48	1.64	2	8.09
Orange II	1.613	3.29	1.714	4.99	2	13.89
Metanil Yellow	1.456	2.23	1.587	3.62	1.91	10.99
Chrysoidine Y	1.415	2.26	1.617	4.62	2	17.09
			pH 7.65			
Congo Red	1.439	1.26	1.615	2.28	2	7.69
Congo Red—com.	1.336	0.96	1.638	2.50	2	7.73
Pontamine Blue BBF	1.754	2.60	1.754	3.18	2	6.72
Mercurochrome	1.765	4.92	1.887	7.56	2	11.82
Orange II	1.626	3.65	1.808	7.16	2	15.43
Metanil Yellow	1.6	3.44	1.719	5.75	2	15.68
Chrysoidine Y	1.746	7.36	1.818	10.01	2	18.95

Even if the conditions upon which Fick's law is based (maintenance of the initial concentration of the diffusing substance and of the cross section in which diffusion is occurring) are observed, deviations have been noticed especially in the diffusion of a colloidal substance within a gel. One explanation[8] which has been offered is that the gel may act as a sieve, holding back larger particles (which may be caused by association of molecules or ions) and permitting the passage of smaller particles. This effect is unlikely in the present

experiments, because deviations are observed with dyes of large and of small molecular weights and because the dye in penetrating into the gel finds the same structure of gel as that in which it was dissolved. Another and more probable cause of the apparent deviations reported in this paper is adsorption of the dye on the fibers of the gelatin. Moravek[11] has measured the penetration of the lead ion into a gelatin gel containing bichromate ion and finds that the results may be expressed by a parabolic equation:

$$a = kt^{\frac{1}{n}}$$

in which a represents the depth of the diffusion layer, t the time, and k and n are constants. He ascribes his results to the affinity of the ion for the gel. It will be seen that the above equation is in agreement with the one we have used.

Friedman[5] has studied the relationship between the molecular weight of nonelectrolytes and their diffusion constants measured in gelatin. When he plots $\frac{1}{\sqrt{M}}$ against k times 10^5, the values fall near a straight line, showing that the size of the molecule influences diffusion to a large extent but that each substance does have some specific effect on the gelatin which prevents the product $k\sqrt{M}$ from being constant. The nonelectrolytes which he investigated had relatively low molecular weights. As the molecular weight and the colloidal nature of the substance increases, it is reasonable that such deviations would become more pronounced. Table IV gives the values $k_3\sqrt{M}$ for the dyes investigated. The constant k_3 was chosen because in this case diffusion was not masked by interfering factors. The symbol M represents the relative weight of the dye ion.

TABLE IV

DYE	M	$\sqrt{M}$	k_3	$k_3\sqrt{M}$
	pH 5.5			
Congo Red	650	25.5	8.19	208.8
Orange II	327	18.08	13.89	251.2
Chrysoidine Y	213	14.6	17.09	249.4
Metanil Yellow	352	18.76	10.99	206.2
	pH 7.65			
Congo Red	650	25.5	7.69	196.1
Pontamine Blue BBF	840	28.98	6.72	194.8
Mercurochrome	707	26.59	11.82	314.3
Orange II	327	18.08	15.43	279.0
Metanil Yellow	352	18.76	15.68	294.1
Chrysoidine Y	213	14.6	18.95	276.6

From a study of the above table one is impressed by the rapid diffusion of mercurochrome. Its molecular weight should indicate slow diffusion and colloidal dispersion. Yet its value for $k_3\sqrt{M}$ shows that it is more closely related to the dyes which are considered to be molecularly dispersed. Teague and Buxton[16] have measured the dialysis of various dyes and find that eosin, which has the same general structure as mercurochrome, can be dialyzed to approximately the same extent as chrysoidine although its molecular weight

is three times as large; congo red, with roughly the same molecular weight as eosin, cannot be dialyzed. These facts are in agreement with the results we have obtained.

SUMMARY

In a dye that is to be used as a stain the rate of diffusion through the tissue is important; in a dye that is depended upon as an antiseptic it is much more so. Obviously sterilization cannot extend beyond the zone of penetration.

Diffusion of dyes in a gel closely duplicates diffusion in tissues. Measurements have been made on the diffusion of various dyes into a 5 per cent gel of 80 per cent ethylene glycol buffered at pH 5.5 and pH 7.65, using a photometer to determine the distance of penetration of definite concentrations of the dye. Differences in diffusion distances of a carefully purified sample and of a commercial sample were found to be negligible when the actual dye content of the two samples was the same. When diffusion was measured to a concentration so small that to visual observation it appeared to be zero, the relationship $k = \frac{d^2}{t}$ was found to give constant values; when diffusion was measured to larger concentrations, constants were obtained for $k = \frac{d^n}{t}$, with n representing some fractional value between 1 and 2. The value of n varied with the dye and with the concentration to which diffusion was progressing; for an individual dye it was usually larger when diffusion to a more dilute concentration was measured. Adsorption of the dye on the fibers of the gelatin is believed to account for the fractional values of n. The rapid diffusion of mercurochrome, in spite of its large molecular weight, is noteworthy.

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SEASONAL VARIATIONS IN SOME PHYSIOLOGIC VARIABLES*

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THROUGHOUT the psychophysiologic study of the child subjects of the Fels Institute it has become increasingly evident that the season of measurement is an important factor influencing the results. It has been shown[10] that, in general, physiologic responses for these subjects were more consistent over a twelve-month period from winter to winter than they were over a six-month period from winter to summer.

It was thought at first that the variability of the summer data might be the result of the unstable temperature which prevails in summer in the area where the measurements were made. It was discovered, however, that although there tends to be some correlation between environmental temperature in summer and response for certain of the variables measured, some subjects were showing consistent variations which were opposite in direction to that shown in the above correlations. It was postulated that true seasonal variations were demonstrated in our data.

These seasonal variations were illustrated for one variable in an article reporting our method of measuring what has been termed "autonomic balance," i.e., a quantitative estimate of the functional state of the autonomic nervous system.[11, p. 433] The autonomic scores for six children over a period of 18 months were shown. Two cases represented those individuals who have higher scores (indicative of parasympathetic predominance) in the winter than in the summer. A second group of four cases illustrated children who show higher scores in the summer than in the winter. Another set of six cases[11, p. 432] indicated that other children may show no marked variation between the two seasons.

Since that report, additional data have been collected during the summer of 1941 and the winter of 1942. The present paper presents an analysis of all the data for those variables involved in the determination of autonomic balance. Measurements of systolic and diastolic blood pressure have also been studied. Specifically, we wish to know (a) whether there are significant group differences between summer and winter measurements for these physiologic

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responses, and (b) whether individuals show consistent differences in direction of seasonal variation.

No extensive reference is made to the literature related to these problems. Earlier work on human subjects has, in most instances, involved two few cases to answer the first question. The controversial results reported on a few cases for such variables as heart rate,[2, 4] respiration rate,[5] and others,[1, 4, 5] may be construed as evidence that the second problem proposed above is deserving of study. Certainly the studies of Peterson,[8] Mills,[6] and Pottenger[9] attest to the importance of the weather as a factor in health and disease, and therefore in general physiologic function.

The data reported herein have been collected in a routine manner by standardized procedures already described.[10] During the winter months of January, February, and March, all of the child subjects of the Institute who are six years of age or older come to the laboratories for a full day of measurement and study. During the summer months of June, July, and August, they again visit the laboratories for periods varying from one day to two weeks. All physiologic measurements are conducted during the hours between 9 A.M. and 12 noon. All but two, salivary output and standing palmar skin conductance, are measured in the resting state. Whenever possible, the recorded datum for an individual represents the mean of a series of samples during the examination period, which lasts about forty-five minutes. The temperature of the measurement room is maintained at 74 to 76° F. during both summer and winter, and the relative humidity is maintained at approximately 40 per cent during the winter and 60 per cent during the summer. For the purposes of this paper this difference in relative humidity is regarded as being a component of the season of measurement.

THE GROUP ANALYSIS

Since the majority of the variables studied bear some relationship to age, it was necessary to eliminate this factor from the analysis. This was done by the simple technique of comparing the means of the data for two (then three) successive winters with the values for the intervening summer (then the mean of two successive summers). The midpoint for chronological age is thus identical for the groups of data compared in each analysis. The first part of Table I shows the means, standard deviations, reliability coefficients, and criteria for judging the significance of the differences between the means for the winters of 1940 and 1941 and the summer of 1940. The data are the results of ten physiologic measurements of fifty-one children ranging in age from 6 to 12 years. The second half of the table shows a similar analysis for twenty-three of those subjects for whom data were available for the summer of 1942. Here, since a smaller number of cases was involved, the standard error of the difference has been based upon the standard deviation of the differences themselves as demonstrated by each subject.*

*The formula for the standard error of the difference between means used in the first analysis is that illustrated by Peters and Van Voorhis[7] as No. 92. For the second analysis, see their formula No. 95. The correlations employed in the first analysis are those obtained for the winter and summer data of 1940. In the second analysis (23 cases) the 1942 winter data for diastolic blood pressure and palmar skin conductance were not included, because part of them were believed to be unreliable. Since one influenced the scores of pulse pressure and both affected the derived scores of autonomic balance, the 1942 winter data for these variables also were omitted from the analysis. Diastolic blood pressure, the only one of these four variables significantly related to chronological age, was corrected for the resulting age discrepancy.

TABLE I

SEASONAL DIFFERENCES BETWEEN MEANS WITH t AND P VALUES OF THE DIFFERENCES FOR TEN PHYSIOLOGIC MEASUREMENTS OF CHILDREN AGED 6 TO 12 YEARS

NO. AND VARIABLES	N = 51								N = 23						
	SUMMER 1940		WINTERS 1940 AND 1941		DIFFERENCE				SUMMERS 1940 AND 1941	WINTERS 1940, 1941 1942	M DIFFERENCES				
	M_1	σ	M_2	σ	R	M_1-M_2	σM_1-M_2	t	P	M_3	M_4	3-4	σD	σM_3-M_4	t	P
23 Dermographia Persistence (minutes)	12.20	9.02	12.67	10.95	.41	−0.47	1.54	0.31	.76	11.78	12.57	−0.79	9.21	1.96	0.40	.69
25 Salivary Output (c.c. per 5 minutes)	4.41	2.46	4.06	2.04	.70	0.35	.25	1.40	.16	4.83	4.57	.26	1.26	.27	0.97	.35
28 Heart Period (seconds)	.745	.05	.723	.086	.66	.022	.009	2.44	.01	.757	.740	.017	.045	.01	1.70	.10
37 Palmar Conductance (microhms)	23.3	6.8	33.5	11.0	.32	−10.2	1.53	6.67	<.01	29.0	33.8	−4.8	9.42	2.01	2.39	.03
39 Volar Conductance	14.2	8.5	14.2	6.3	.12	0.0	1.39	0.0		11.4	13.3	−1.9	5.41	1.15	1.65	.12
57 Respiration Period (seconds)	3.01	1.95	3.19	1.04	.37	−0.18	.26	0.69	.49	2.99	3.18	−0.19	.518	.119	1.73	.10
76 Systolic Blood Pressure (mm. Hg)	96.20	6.40	98.50	5.99	.70	−2.30	.67	3.43	<.01	96.48	99.48	−3.00	3.47	.740	4.05	<.01
77 Diastolic Blood Pressure	60.70	6.74	61.60	7.45	.55	−0.90	.95	0.95	.34	59.96	60.35	−0.39	5.59	1.19	0.33	.72
80 Pulse Pressure	35.50	5.96	36.50	8.26	.38	−1.00	1.14	0.88	.38	35.17	37.30	−2.13	5.20	1.11	1.92	.07
A Autonomic Balance	72.50	9.03	72.65	7.97	.57	−0.15	1.11	0.14	.88	50.78	51.65	−0.87	6.17	1.32	0.66	.52

It will be seen that both analyses agree in placing the seasonal difference in systolic blood pressure at better than the .01 level of significance. The probability is almost as remote that the difference in palmar skin conductance could have occurred by chance; and the chances are high that the seasonal change in heart period also represents a true difference. Although the level of significance for none of the other measures is as high in either analysis as .05, it is of interest that both analyses agree in the direction of the differences.

From these results the first question may be answered. The probability is great that during the summer, as compared with the winter, systolic blood pressure is lower, standing palmar skin conductance (palmar sweating) is lower, and the heart beats at a slower rate. There also is some indication that salivary output is greater, respiration rate is faster, and pulse pressure is lower. The chances are low that true differences obtain for diastolic blood pressure, volar skin conductance, dermographia persistence, or autonomic balance, although the differences found here for these four variables are in the same direction in both analyses. The small change in the estimates of autonomic balance is of particular interest, as will be made clear in the following discussion.

For convenience in interpreting the results the 1940 regression equation[12] for the estimation of autonomic function is reproduced below. This equation is used because it is believed to be more representative of autonomic function than the 1941 equation, scores on which are greatly influenced by individual deviations in heart period.

$$\overline{Nu} = .15z_{23} + .39z_{25} + .31z_{28} + .38z_{37x} + .10z_{39x} - .10z_{57} + .22z_{80}$$

In the equation, $\overline{Nu}$ signifies the estimate of autonomic balance for a given individual; z equals the individual's standard score in the trait designated by the number in subscript; and x, following trait numbers 37 and 39, indicates that these traits have been reflected so that a high standard score is the equivalent of a low raw score. The names of the traits are the same as shown in Table I. A high score derived by means of this equation is considered indicative of functional predominance of the parasympathetic branch. A homogeneous shift in all functions toward the parasympathetic end of the scale would involve a lengthened persistence of red dermographia, an increased salivary output, a slowed heart rate, decreased palmar and volar conductance or sweating, an increased respiration rate, and a heightened pulse pressure. With the exception of dermographia persistence and pulse pressure, this is the pattern of response for summer (shown in Table I). Since these two variables are not heavily weighted in the equation, it might be expected that seasonal comparisons of these scores would reveal a significant difference between summer and winter scores of autonomic balance, with the summer season effecting a shift toward the parasympathetic end of the scale. Such a shift might also be predicted from the findings of Gellhorn and Feldman,[3] who report that heat, although affecting both the vago-insulin and sympathetico-adrenal systems, has its greatest effect upon the former. Table I shows, however, that no reliable difference obtains. The next section of this report will deal further with this finding.

THE PROFILES FOR 23 INDIVIDUALS

The individual data for the 23 children who had had five consecutive measurements were analyzed in the same general manner as were the group data for 51 cases. The scores for a given subject were combined in three ways, two combinations being the mean of two adjacent winters and the intervening summer, and the third being the mean of the two summers and the intervening winter. If the summer score for a given variable was higher than the winter score *for all three combinations*, the child was scored plus (+) for that variable. If the winter score was higher than the summer score *for all three combinations*, the child was scored minus (−). If the combinations differed in direction of change, no symbol was assigned. Instances of "no change" were infrequent and, when occurring among otherwise consistent changes, were disregarded. The series of 23 profiles obtained in this manner are shown in Table II. It should be noted that four of the variables have been reflected to make their direction consistent with respect to autonomic activity.

It will be seen that the cases are grouped in four categories. Since systolic blood pressure and palmar skin conductance had shown the most significant seasonal variation, it was argued that consistent variation in either of these variables in a direction opposite to that shown by the group might be considered as of possible significance. As might be expected, the largest group, comprising nine cases, is that which shows during the summer measurements a lower systolic blood pressure *and* a lower palmar conductance. Group II contains the five subjects who manifest higher systolic blood pressure in summer. Group III comprises four children who show higher palmar conductance in summer than in winter. Group IV includes five cases concerning which the criterion data are indecisive. The criterion data are underlined in Table II.

It is of interest to note that heart rate is slower in summer for Group I, faster in summer for Group II, and inconsistent in change for Group III. Pulse pressure also shows a certain differential group consistency. In general, it appears to be decreased in summer in Group I and increased in Groups II and III. Diastolic pressure tends to show the opposite picture. Although the results are not decisive, dermographia persistence time seems shorter in summer for Groups II and III, while volar skin conductance is lower in summer for Group I. The groups are not well differentiated for the variables salivary output or respiration period.

The first seven variables of Table II are those used in the estimation of "autonomic balance." With the reflection of the skin conductance variables 37 and 39, positive signs for all variables indicate a shift toward greater parasympathetic function; conversely, negative signs indicate a shift toward functional predominance of the sympathetic system. While there is some suggestion that Group I shows a majority of positive symbols, and Groups II and III, a majority of negative signs, a more meaningful interpretation may be drawn from the last row of the table showing the direction in shift of the derived autonomic scores. Here Groups II and III are seen to contain no positive signs. Group I contains a majority of positive signs in the last row, and the data offer a ready explanation for the three negative symbols, the encircled symbols for

TABLE II

PROFILES FOR 23 CHILDREN SHOWING THE DIRECTION OF CONSISTENT SEASONAL VARIATIONS IN TEN PHYSIOLOGIC VARIABLES OVER THREE WINTERS AND TWO INTERVENING SUMMERS*

NO. AND VARIABLE		GROUP I										GROUP II					GROUP III				GROUP IV				
	CASE NO. AND SEX	3F	4M	17F	75F	77M	87M	88F	91F	105F		20M	73F	81M	83F	86F	28F	74M	82F	92M	41F	42F	67F	85M	102M
23	Dermographia Persistence (minutes)	+		−	−			−	+	+					−	−	+	−	−	−	−		−	+	
25	Salivary Output (c.c. per 5 min.)		⊖	+	⊖	−	+	+		−		−	−	−				+	+				+		−
28	Heart Period (seconds)	+	+	+	+		+	⊖	+	+		−	−		−	−						−			+
37x	Palmar Conductance (microhms)	+	+	+	+	+	+	+	+	+		+	+		+	+	−	−	−	−	+	+	+		+
39x	Volar Conductance	+	−	+			+	+	+	+		−		−	+			+		−		+	+		
57	Respiration Period (seconds)	+					−	−		−											+	−	−		+
80	Pulse Pressure		−	−			−	−	+	−			+	+	+	+		+	+				−	+	−
76x	Systolic Blood Pressure (mm. Hg)	+	+	+	+	+	+	+	+	+		−	−	−	−	−	+	+	−	+					
77x	Diastolic Blood Pressure	+				+		−	+	−		−	+	+	+	+		+	+		−		−		−
A	Autonomic Balance	+	−	+	−	+	+	−	+	+		−	0	−	−	−	−	−	−	−	−	−	+	+	−

* + signifies consistently higher scores in summer than in winter.
− signifies consistently lower scores in summer than in winter.
x indicates reflection of variable.

the heavily weighted variables of salivary output and heart rate. Perhaps these encircled symbols represent experimental artifacts or discrete functional differences apart from the total variance in autonomic function.

In terms of this latter method of differentiation these subjects might be reclassified into two groups. Six cases from Group I and two from the indeterminate Group IV comprise those individuals who show a relatively greater parasympathetic function in summer than in winter (Group +). Groups II and III and the remainder of the cases of Groups I and IV are those who manifest relatively greater sympathetic function in summer (Group −). Since the two groups are rather evenly divided, the explanation for the lack of a reliable total group seasonal variation becomes apparent. It is of further interest that the two groups differ significantly in mean winter autonomic score. The mean winter autonomic score for Group + is 69.4. The mean for Group − (omitting the one subject who shows no variation from summer to winter) is 77.5, or approximately one σ higher. Since the σ of the difference in means is only 3.14, the obtained t of 2.58 may be considered as significant at approximately the 0.04 level of probability, when only 7 degrees of freedom are allowed. Apparently the summer season tends to effect a shift toward greater parasympathetic activity in those individuals having low autonomic scores (apparent predominance of sympathetic function) in winter, and a shift toward greater sympathetic activity in those individuals demonstrating high autonomic scores (apparent predominance of parasympathetic function) in winter. The summer seasons seem, then, to have a levening effect upon autonomic function, and it seems pertinent to question whether summer might not afford more "basal" conditions for the determination of scores of autonomic balance. Scores obtained in winter might then be viewed as reflecting the reaction of the organism to a prolonged polar front. In most temperate latitudes, however, winter must remain the season of choice for such determinations, for the environmental temperatures during the summer months are not sufficiently stable to permit the collection of comparable data on a large group.

It remains to be determined whether or not this differential seasonal variation is significant in behavior and disease. The results certainly are consistent with the observations of Peterson,[8] Mills,[6] and Pottenger.[9] Furthermore, the different groupings of seasonal variations in different functions serve to re-emphasize a consideration that Peterson has stressed; namely, the necessity for considering individual differences in response to climatic changes in any study of physiologic function.

SUMMARY

This study has concerned itself with two questions: (a) Are there significant group differences between summer and winter measurements of certain physiologic responses. (b) Do individuals show consistent differences in direction of seasonal variations. Two analyses of group data have been made; one involving data obtained during two consecutive winters and the intervening summer for 51 children 6 to 12 years of age, and the other involving data obtained during an additional winter and summer for 23 of the same children. Individual profiles were shown for these 23 cases.

The two group analyses agree in finding systolic blood pressure and palmar skin conductance (palmar sweating) significantly lower during the summer measurements. The chances are high that heart period also is significantly longer in summer. Although none of the other differences found may be regarded as reliable, there is some indication that in summer as compared with winter, diastolic blood pressure and pulse pressure are lower, salivary output is greater, respiration is faster, dermographia persistence time is shorter, and volar conductance or sweating is decreased.

The analysis of individual profiles shows that the seasons consistently have different effects on different individuals for each of the variables studied. It is believed that individuals may be meaningfully classified in at least two groups in terms of scores of autonomic balance: those who manifest relatively greater parasympathetic function in summer than in winter, and those who demonstrate relatively greater sympathetic function in summer than in winter.

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CLINICAL CHEMISTRY

THE EFFECT OF CONTINUED SULFANILAMIDE INGESTION ON THE ACID-BASE EQUILIBRIUM OF THE DOG*

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A NUMBER of studies have been made of the effect of sulfanilamide on the acid-base equilibrium of man and animals.[2-10] Some of these studies have been carried out in patients in whom complicating effects were present. Others have either employed quantities of sulfonamide outside the range that is considered therapeutically effective or have been confined to observations following single doses of the drug. The general results of these studies have indicated that in some patients and animals evidence of acidosis is present following sulfanilamide ingestion. As a result of the effects that arise from sulfanilamide, the use of alkali with the compound has been suggested and has been rather widely used.

The present report describes studies conducted with normal dogs which were given sulfanilamide either alone or accompanied by sodium citrate. The purposes of these studies were: (1) to observe the changes in the acid-base equilibrium of the blood following the ingestion of quantities of sulfanilamide sufficient to maintain therapeutic levels in the blood; (2) to determine the changes in the acid-base equilibrium of the blood produced by combinations of sodium citrate and sulfanilamide; (3) to ascertain whether the restriction of dietary salt intake would influence the effects of sulfanilamide with or without alkali; (4) to observe the effect on the acid-base equilibrium of the blood when the concentrations of sulfanilamide are maintained above therapeutic levels over a period of several days; (5) to determine the effect of single massive doses of sulfanilamide; (6) to examine the changes in urine pH during the maintenance of high levels of blood sulfanilamide.

METHODS

Adult female dogs were employed in all of the studies and were maintained on a diet of Purina dog chow except during the studies with the low salt diet. This low salt diet was similar to that employed by Nilson,[11] consisting of cracker meal, cane sugar, casein, butterfat, and lard, and having a very low content of sodium, potassium, and chlorine. Blood samples were obtained from the jugular veins with a minimum of stasis, and urine samples were obtained by catheteriza-

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tion. Methods of handling the blood as well as the references to the chemical methods employed are reported elsewhere.[10] Hemoglobin determinations were carried out on whole blood by the method of Evelyn,[12] while red and white cell counts were made by conventional hematologic techniques.

CHANGES OBSERVED IN THE ACID-BASE BALANCE OF THE BLOOD

Animals Receiving a Normal Diet.—In this series of experiments the dogs were given sulfanilamide alone, sulfanilamide and sodium citrate, or sodium citrate alone while ingesting a normal diet. Three dogs were included in each group and, by shifting the animals, the same three dogs acted as subjects in each group. Approximately ten to twelve days elapsed between the end of one experiment and the beginning of the next. The routine used in these experiments was as follows: A fasting blood sample was obtained at 8:30 A.M. on the first day of the experiment, and this was subjected to a "complete analysis" which included determinations of serum pH, carbon dioxide content, chloride, inorganic phosphorus, protein, and total base and whole blood determinations of hemoglobin, sulfanilamide, and red and white cell counts. A sample of urine, obtained by catheterization, was also taken at this time and its pH determined. At 9:00 A.M. each dog received by stomach tube the indicated compound or mixture in an aqueous solution of approximately 500 c.c. The initial dose was twice as large as all the succeeding doses. For this reason the intake of the compounds in question is 33 per cent higher on the first day than on all the following days. A second dose of the drug was administered at 1:00 P.M. and a third dose, at 5:00 P.M. The total daily dose (on all except the first day) of sulfanilamide was 0.20 Gm. per kilogram of body weight, and the dose of sodium citrate was 1.0 Gm. per kilogram. Each single dose represented one third of the daily dose. An afternoon sample of blood was taken at 4:00 P.M. and submitted to "partial analysis," consisting of determinations of serum pH, carbon dioxode content, chloride, and blood sulfanilamide. An afternoon (4:00 P.M.) urine specimen was obtained by catheterization and the pH determined. On the succeeding days of the experiment the same schedule was followed and a "partial analysis" was made on the morning and afternoon blood. Cell counts and hemoglobin estimations were done each morning, and morning and afternoon urine samples were obtained by catheterization for pH estimations. On the morning of the day that the drug was discontinued a sample of blood for "complete analysis" was again obtained.

The results of this experiment are presented in the first four columns of Table I. By the nature of the experiment, the morning analysis represents the blood level at a time fifteen hours after the preceding dose of drug, whereas the afternoon analysis represents the blood level three hours after the preceding dose. During the experiment no trend of change from day to day was discernible, and consequently only the average values for the morning and afternoon samples during the experimental period are recorded. From the data presented in Table I it will be seen that considerable fluctuation in blood sulfanilamide level occurred between the morning and afternoon samples as was to be expected. On the other hand, the level of blood sulfanilamide was approximately the same after sulfanilamide and sodium citrate as after sulfanila-

mide alone. It will be seen that the serum pH was not appreciably altered from the control level by sulfanilamide, by sulfanilamide and sodium citrate, or by sodium citrate alone, and, furthermore, there were essentially no differences in the morning and afternoon samples. The serum chloride was maintained at approximately control levels in all of the present experiments. Serum bicarbonate was slightly decreased after sulfanilamide alone, but in the afternoon samples, three hours after the administration of sulfanilamide and sodium citrate or sodium citrate alone, the serum bicarbonate was increased. The hemoglobin content of the blood was not significantly altered in any of the experiments, the slight variations encountered probably being explained by the loss of the blood removed for the chemical analyses. The erythrocyte and leucocyte counts of the blood were not appreciably changed. No significant changes in total base, protein, phosphate, or undetermined acid were noted between the beginning and end of the experiment.

Animals Receiving a Low Salt Diet.—In this series of experiments, dogs were maintained on a synthetic diet quite low in chloride and total base and were given the same combinations of sulfanilamide and sodium citrate that were employed in the preceding experiments. The low salt diet was given for one week before the start of the study and was continued during the interval of eight days between experiments. Each of the three dogs under observation was shifted so that each served in each group. The routine followed in these experiments was exactly the same as that used in the above studies on the normal salt diet with the exception that no blood or urine was taken on the third and fifth days, although the administration of the drugs was the same on these days. The results of this experiment are indicated in the last four columns of Table I. It will be seen that, in general, the results of this experiment are similar to those obtained when the animals received a diet with a normal salt content. However, throughout the experiment there was a tendency to slightly greater changes in pH and bicarbonate as a result of the ingestion of sodium citrate either alone or with sulfanilamide.

Animals Receiving Sulfanilamide at Four-Hour Intervals.—In order to ascertain the effects of blood concentrations maintained somewhat above the therapeutic levels, and also in order to decrease fluctuations of blood sulfanilamide concentrations, sulfanilamide was administered six times per day at four-hour intervals. The drug was given at 9:00 A.M., 1:00 P.M., 5:00 P.M., 9:00 P.M., 1:00 A.M., and 5:00 A.M. in amounts of 0.05 Gm. per kilogram of body weight per dose. Blood samples were obtained twice each day at 8:00 A.M and 4:00 P.M. Urine samples were obtained at four-hour intervals (see below). Three dogs were studied in this way, the sulfanilamide being given for four, six, and seven days to the three animals. The analyses included blood sulfanilamide and serum pH, bicarbonate, and chloride determinations. The results of these studies are summarized in Table II. Dogs 1 and 3 did not exhibit any adverse effects from this experiment, whereas Dog 2, after the first day of the experiment, completely refused all food and vomited considerably during the period of sulfanilamide ingestion. It will be noted that the blood sulfanilamide concentration in this dog reached higher levels than in the other two dogs and a progressive increase occurred during the latter part of the experiment. At the

TABLE I

AVERAGE BLOOD FINDINGS* DURING CONTINUED ADMINISTRATION OF SULFANILAMIDE AND ALKALI

	NORMAL DIET				LOW SALT DIET			
	CONTROL	DURING ADMINISTRATION OF SULFANILAMIDE†	DURING ADMINISTRATION OF SULFANILAMIDE AND SODIUM CITRATE‡	DURING ADMINISTRATION OF SODIUM CITRATE§	CONTROL	DURING ADMINISTRATION OF SULFANILAMIDE†	DURING ADMINISTRATION OF SULFANILAMIDE AND SODIUM CITRATE‡	DURING ADMINISTRATION OF SODIUM CITRATE§
Blood sulfanilamide A.M. (mg. per 100 c.c.)	0.0	7.9	8.6	0.0	0.0	8.3	7.6	0.0
Blood sulfanilamide P.M. (mg. per 100 c.c.)	0.0	15.8	17.4	0.0	0.0	15.5	13.4	0.0
Serum pH (A.M.)	7.40	7.40	7.42	7.40	7.38	7.39	7.41	7.41
Serum pH (P.M.)	7.41	7.41	7.43	7.42	7.38	7.41	7.43	7.44
Serum chloride A.M. (meq. per L.)	109.2	110.8	110.3	106.5	109.3	107.9	105.8	105.0
Serum chloride P.M. (meq. per L.)	109.2	110.2	108.5	105.3	109.3	107.3	102.6	102.1
Serum bicarbonate A.M. (meq. per L.)	24.1	22.2	24.9	25.0	24.3	21.9	25.2	26.5
Serum bicarbonate P.M. (meq. per L.)	24.1	22.3	27.0	29.0	24.3	22.3	28.7	31.5
Serum bicarbonate (P.M.) (meq. per L.)	12.4	11.3	11.7	12.4	13.5	13.4	12.3	13.4
Hemoglobin (Gm. per 100 c.c.)	5.7	5.3	5.3	5.5	5.8	5.5	5.6	5.9
Erythrocyte count (million per cubic millimeter)	8.7	10.4	9.9	11.0	14.0	**11.5**	12.4	11.9
Leucocyte count (thousand per cubic millimeter)								

*Blood taken for analysis twice daily, at 8:00 A.M. and 4:00 P.M.

†0.20 Gm. of sulfanilamide per kilogram of body weight per day, administered in three doses at 9:00 A.M., 1:00 P.M., and 5:00 P.M. each day for a period of six days to three dogs.

‡0.20 Gm. of sulfanilamide per kilogram per day plus 1.0 Gm. of sodium citrate per kilogram per day administered in three doses at 9:00 A.M., 1:00 P.M. and 5:00 P.M. each day for a period of six days to 3 dogs.

§1.0 Gm. of sodium citrate per kilogram per day administered in three doses at 9:00 A.M., 1:00 P.M., and 5:00 P.M. each day for a period of six days to three dogs.

same time the serum chloride in this animal fell from an average control value of 110.3 meq. per liter to a minimum of 83.0 meq. the last day of the experiment. At this time the total base concentration of the serum showed a deficit of the same magnitude as the chloride decrease. However, even though this animal was severely dehydrated, there were no significant changes in the serum pH or the serum bicarbonate concentration. It will be seen that some decrease in serum pH occurred in all three dogs, although the decrease was not progressive. Dog 3 showed a somewhat greater lowering of serum pH than the other two dogs and had a minimum value of 7.32 in one of the samples. The serum bicarbonate content showed some fall in all three animals, although the change was not marked in any.

TABLE II

BLOOD STUDIES IN DOGS RECEIVING SULFANILAMIDE AT FOUR-HOUR INTERVALS

	DOG 1	DOG 2	DOG 3
Average blood sulfanilamide (mg. per 100 c.c.)	30.4	47.5	21.4
Maximum blood sulfanilamide (mg. per 100 c.c.)	43.5	70.8	33.1
Control serum pH	7.40	7.40	7.41
Average experimental* serum pH	7.39	7.37	7.35
Minimum experimental* serum pH	7.35	7.35	7.32
Control serum chloride (meq. per L.)	110.2	110.3	112.0
Average experimental* serum chloride (meq. per L.)	112.4	99.8	110.0
Minimum experimental* serum chloride (meq. per L.)	109.0	83.0	107.0
Control serum bicarbonate (meq. per L.)	25.7	24.7	22.5
Average experimental serum bicarbonate (meq. per L.)	21.3	21.4	20.6
Minimum experimental serum bicarbonate (meq. per L.)	19.3	19.6	19.1

*During sulfanilamide administration.

Animals Receiving a Single Large Dose of Sulfanilamide.—In order to ascertain the effect of large quantities of sulfanilamide on the acid-base equilibrium, dogs were given a single large dose of 1.0 Gm. of sulfanilamide per kilogram of body weight. The sulfanilamide was mixed with approximately 500 c.c. of warm water and was given as an aqueous suspension. With this quantity of sulfanilamide, marked dyspnea developed in all three animals, accompanied by clonic convulsions, spasticity, extreme salivation, and loss of most reflexes. A control blood sample was obtained along with samples at one, three, six, twelve, twenty-four, and forty-eight hours after the administration of the drug. Chemical analyses of the blood included those determinations recorded in Table III. It will be observed that a maximum concentration of blood sulfanilamide was obtained in the three-hour sample, although the concentration in the six-hour sample was quite similar. The values presented in Table III indicate that there were no marked changes in the acid-base equilibrium of the blood, although all of the animals showed marked dysfunction of the central nervous system. Hemoglobin and serum protein concentrations showed elevations that were a maximum at six hours, which would indicate that there was a loss of water from the blood.

CHANGES OBSERVED IN THE URINE pH

In both the experiments with a normal diet and with the low salt diet, it was found that the pH of urine samples obtained by catheterization were acid in the morning and alkaline in the afternoon. It has been suggested that sulfanilamide has some specific effect in preventing bicarbonate reabsorption in the

kidney tubules and that this is the basis for the disturbance in the acid-base equilibrium which follows the ingestion of sulfanilamide. In the experiments with the normal diet and the low salt diet, the blood level of sulfanilamide was at the low point when the urine was acid and was considerably higher when the urine was alkaline. The urine pH was studied more closely in the animals receiving sulfanilamide at four-hour intervals. The routine followed in these experiments has been described above, and it is to be noted that urine samples were obtained by catheterization at four-hour intervals during the day and night. The urine was collected just prior to sulfanilamide administration. With two or three exceptions, the dogs did not urinate between the four-hour catheterizations, so the entire urine output was collected in this manner. The maximum and minimum values of urine pH for the three animals for each of the days that they were receiving sulfanilamide are given in Table IV. It will be observed that a considerable range of urinary pH was encountered during the

TABLE III

BLOOD STUDIES FOLLOWING A SINGLE LARGE DOSE* OF SULFANILAMIDE
AVERAGE RESULTS OBTAINED WITH THREE DOGS

	CONTROL	1 HR.	3 HR.	6 HR.	12 HR.	24 HR.	48 HR.
Blood sulfanilamide (mg. per 100 c.c.)		57.8	67.0	62.0	37.7	18.7	
Serum pH	7.39	7.38	7.37	7.37	7.36	7.36	7.36
Serum total base (meq. per L.)	160.2	157.9	160.2	164.1	165.7	155.8	
Serum chloride (meq. per L.)	110.8	107.7	108.7	110.0	108.5	109.7	108.6
Serum bicarbonate	22.8	21.4	19.6	20.2	19.1	19.4	20.4
B Pr,† (meq. per L.)	14.1	14.8	15.3	16.8	16.5	15.2	
B $PO_4^{\ddagger}$ (meq. per L.)	4.1	4.3	3.8	3.2	5.6	4.1	
Undetermined acid (meq. per L.)	8.4	9.7	12.8	13.9	16.0	7.4	
Hemoglobin (Gm. per 100 c.c.)	12.3	12.8	15.2	16.0	13.7	12.0	
Urine pH	7.4	7.4	7.8	7.9	7.6	7.0	7.0

*1.0 Gm. of sulfanilamide per kilogram of body weight.
†Base combined with protein.
‡Base combined with phosphate.

TABLE IV

VARIATION IN URINE pH IN DOGS RECEIVING SULFANILAMIDE

EXPERIMENTAL DAY		DOG 1	DOG 2	DOG 3
1	Max.	8.4	7.6	7.0
	Min.	7.0	5.9	5.5
2	Max.	7.8	8.1	7.6
	Min.	6.5	5.9	6.2
3	Max.	7.8	7.4	7.5
	Min.	6.6	5.5	6.1
4	Max.	7.6	7.3	7.6
	Min.	6.6	5.2	5.6
5	Max.	7.5	7.4	
	Min.	5.9	6.9	
6	Max.	7.4	7.2	
	Min.	6.1	6.1	
7	Max.		7.2	
	Min.		6.2	

same day in all of the dogs. With but two exceptions, the maximum pH was on the alkaline side of neutrality while the minimum pH was on the acid side of neutrality. The two exceptions mentioned occurred in Dogs 1 and 3 on the first experimental day. Dog 1 on this day had a maximum pH of 8.4 and a minimum pH of 7.0, whereas Dog 3 had a maximum pH of 7.0 and a minimum pH of 5.5. The only instance in which the fluctuation in pH was less than 1.0 occurred in Dog 2 on the fifth experimental day, when the maximum pH value was 7.4 and the minimum value was 6.9.

DISCUSSION

The present studies indicate that, with continued sulfanilamide administration and the maintenance of therapeutic levels of sulfanilamide in the blood, there are no great changes in the acid-base equilibrium. The small decrease in serum bicarbonate that occurs can be prevented by the administration of sodium citrate along with the sulfanilamide. Hematologic studies in the dogs receiving either sulfanilamide alone or sulfanilamide plus sodium citrate indicate that there was no anemia or leucopenia in any of the animals.

Hartmann[6] has suggested that respiratory alkalosis develops following sulfanilamide therapy and that administration of alkali is undesirable since it would tend to accentuate the alkalosis. Hartmann furthermore suggested that chloride deprivation would result in even more marked changes when alkali was given with the sulfanilamide. The present findings in the dogs maintained on a low salt diet do not show any indication that ingestion of sulfanilamide or sulfanilamide and alkali during salt deprivation causes alkalosis, although the urinary excretion of chloride and total base was essentially zero during sulfanilamide administration.

In the experiments in which animals were given sulfanilamide every four hours, there is further evidence to indicate that sulfanilamide does not cause severe disturbance in the acid-base equilibrium, although in these experiments the average blood level was considerably above that usually sought in clinical practice. In one of the animals (Dog 2) in which a marked dehydration developed as a result of vomiting, the serum chloride and serum total base concentrations fell by approximately 30 meq. per liter; however, the serum bicarbonate and serum pH were not markedly changed. In this experiment the collection of four-hour urine samples indicated that even though relatively high and constant blood sulfanilamide levels are maintained, the urine pH will still show considerable fluctuation. Marshall, Cutting, and Emerson[3] suggested that the excretion of an alkaline urine after sulfanilamide is due to the effect of sulfanilamide in preventing a reabsorption of bicarbonate in the kidney tubules. McChesny, Sprague, and Marshall[9] also believed this to be the case. On the other hand, Höber[13] has suggested that sulfanilamide causes an alkaline urine by reason of its inactivating effect on carbonic anhydrase. From the results that are summarized in Table IV, it would appear that other factors are also involved in regulating urine pH and that the secretion of an alkaline urine will not continue even though the blood sulfanilamide is maintained at a high and constant level. The acid-base equilibrium of the blood did not suggest any basis for the variation in urinary pH.

Marshall, Cutting, and Emerson[3] reported acid-base studies in three dogs that were given large doses of sulfanilamide. The findings suggested acidosis in some of the samples, although the results were not uniform. The dogs reported in Marshall's study showed marked signs of intoxication, as did those described in the present report which were given a single large dose of sulfanilamide. However, careful study of multiple blood samples in all three of the animals of the present study did not disclose any evidence of marked acidosis, although the serum bicarbonate was somewhat decreased. The marked dysfunction of the gastrointestinal tract and respiratory system that occurs following large doses of sulfanilamide gives a basis for disturbances in the acid-base equilibrium of the blood, although in the present experiments no marked disturbances were observed.

All of the present experiments offer evidence that the administration of sodium citrate along with sulfanilamide has no contraindications and may furthermore be desirable from the standpoint of preventing the decrease in serum bicarbonate that follows sulfanilamide ingestion.

SUMMARY

1. Studies of the acid-base balance of the blood were carried out on dogs during continued ingestion of sulfanilamide in amounts sufficient to maintain therapeutic blood concentrations. Small decreases in serum bicarbonate were observed, but there was no appreciable alteration in the serum pH.

2. Administration of sodium citrate along with the sulfanilamide prevented any decrease in serum bicarbonate and did not cause the pH to vary appreciably from the normal.

3. Ingestion of a low salt diet before and during a period when sulfanilamide or sulfanilamide and sodium citrate was ingested did not give results significantly different than were obtained during ingestion of a normal diet.

4. Maintenance of blood sulfanilamide concentrations at levels above those sought therapeutically did not produce any marked changes in the acid-base equilibrium of the blood.

5. Single massive doses of sulfanilamide, sufficient to produce signs of severe intoxication, did not cause marked changes in the acid-base equilibrium of the blood but did tend to cause a loss of water from the blood.

6. The pH of urine samples obtained at four-hour intervals during the maintenance of high blood sulfanilamide levels showed marked daily fluctuations which could not be correlated with certain views expressed in the literature.

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PROTEIN HYDROLYSATE IN THE REGENERATION OF SERUM PROTEIN IN THE HYPOPROTEINEMIC RAT*

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RECENTLY there have appeared reports on the regeneration of various proteins in variously depleted animals. Some of these studies have had the aim of comparing high protein diets with equivalent amounts of protein hydrolysates[1] or of determining the relative efficiency of oral and intravenous administration.[1-3] The results of these investigations have shown, first, that hydrolysates are at least as efficient as whole protein; e.g., in the regeneration of plasma protein in dogs rendered hypoproteinemic by restricted diet,[1] and, second, that hydrolysates are as effective intravenously as orally.[1-3]

In the normal clinical treatment of hypoproteinemic patients (e.g., postoperative care, after more or less severe depletion) it is now not uncommon (for examples see ref. 4) to supplement a regular hospital diet with some protein hydrolysate, possibly an enzymatic digest administered orally (more rarely, and with attendant danger of shock,[3] parenterally) or an acid hydrolysate (fortified, perhaps, with tryptophane and cystine) injected intravenously. Accordingly, experiments were undertaken in this laboratory to determine whether or not a normal diet supplemented by an enzymatic protein hydrolysate would be superior to an unsupplemented diet in the regeneration of serum protein in hypoproteinemic albino rats.

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EXPERIMENTAL

Two sets of litter mates were used in the experiments. The first group, all males, weaned four weeks after the litter was cast, was kept on a diet of Purina Checkers until almost five months old; the second group, two males and four females (segregated as to sex after weaning), were similarly treated until almost four months old.

At this point both litters were put on Diet 6A (Table I). This diet, designed to deplete protein reserves, was fed *ad libitum*. Litter I was kept on this diet for 39 days; Litter II for 26 days. The animals at this time were distinctly hypoproteinemic and had lost much weight (cf. Table II). Each litter was then divided into two groups. One group received Regeneration Diet I (powdered Purina Checkers, total nitrogen 4.29 per cent), and the other, Regeneration Diet II (powdered Checkers (90 per cent) to which was added 5.5 per cent of a protein hydrolysate preparation* and 4.5 per cent cornstarch, total nitrogen 4.27 per cent). This latter mixture contained 10 per cent of its nitrogen in the form of the protein hydrolysate preparation.

On the protein regeneration diets serum protein levels were followed at approximately two-week intervals for Litter I during seven months, for Litter II during 4.5 months. The animals were weighed twice each week.

TABLE I

PROTEIN DEPLETION DIET 6A

Butter (salt)	8.95%
Lard	4.48%
Yeast	0.28%
Cod-liver Oil	2.24%
Osborne-Mendel Salt Mixture	4.48%
Cornstarch	67.25%
Sucrose	12.32%

TABLE II

WEIGHT AND SERUM PROTEIN VALUES

	LITTER I*					LITTER II†				
	GROUP A			GROUP B		GROUP A			GROUP B	
	AGE (DAYS)	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %	AGE (DAYS)	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %	AV. WEIGHT (GM.)	AV. SERUM PROTEIN %
Predepletion period	149	254	6.08	240	6.32	118	177	6.50	181	6.56
End of depletion period	188	166	4.99	162	4.90	144	113	5.43	117	5.35
Regeneration period	203	190	5.49	199	5.79	167	163	5.86	171	6.20
	217	203	5.61	230	5.87	182	177	5.82	187	6.24
	246	232	6.23	231	6.76	217	207	6.04	211	6.48
	318	252	6.14	268	6.33	273	209	6.42	221	7.16
	401	307	6.21	313	6.48	317	210	6.44	222	6.89

*Litter I—Group A, 2 males
Group B, 3 males

†Litter II, Each group, one male and two females
Groups A were on Regeneration Diet I
Groups B on Regeneration Diet II.

*The protein hydrolysate preparation used was Aminoids, a product containing 44.5 per cent of an enzymatic digest of dried powdered beef (15.6 per cent), wheat gluten (41.6 per cent), dried skim milk (41.6 per cent), and dried brewer's bottom yeast (1.2 per cent).

Serum protein was determined by the falling drop method, using a Kagan proteinometer,[5] on serum obtained from tail slices. All determinations were made in duplicate. In Table II are presented some of the data accumulated during these experiments. Full tabulations are omitted to conserve space.

The total consumption of Regeneration Diets I and II was measured, and the average food intake per rat was calculated from these figures. These data appear in Table III.

TABLE III

TOTAL AVERAGE DAILY FOOD CONSUMPTION PER RAT DURING REGENERATION PERIOD

	GROUP A	GROUP B
Litter I	14.35 Gm. (0.615 Gm. N)	14.42 Gm. (0.56 Gm. N)
Litter II	12.88 Gm. (0.552 Gm. N)	13.12 Gm. (0.560 Gm. N)

RESULTS AND DISCUSSION

In the case of Litter I where an average weight loss of 33.5 per cent and an average decrease of 20.4 per cent in blood serum protein concentration were recorded during the protein depletion period, the animals on Regeneration Diet I (without added hydrolysate) required a significantly longer time (123 days as opposed to 100 days) to recover the weight lost. With regard to the regeneration of serum protein, no attempt was made to estimate the amount of new serum protein formed, since blood volume studies were not undertaken. Using as an index the per cent of protein in the blood serum, it will be seen that this value rose at a significantly higher rate during the first 58 days, at least, for the animals on Regeneration Diet II (containing added hydrolysate).

The animals of Litter II showed slightly more severe weight loss (35.7 per cent) and slightly less decrease in per cent of protein in blood serum (17.5 per cent) at the end of the depletion period. Here, however, the weight recovery on both diets took place much more quickly (38 and 35 days on Regeneration Diets I and II, respectively). The only explanation which can be put forth from the data at hand seems to be that although the percentage decrease in blood serum protein content was almost as high as in Litter I, the actual value attained at the end of the depletion period was significantly higher, which suggests that these animals having been less hypoproteinemic were, consequently, in better general health. Also the animals in Litter II were about one month younger than those in Litter I and may still have been in the period of normal growth. Consideration of these factors makes even more striking the fact that the percentage of protein in the blood serum increased more rapidly on Regeneration Diet II than on Diet I.

Especially significant is the response recorded in the blood serum protein levels during the first intervals in each experiment. In Litter I the increases on Diets I and II were respectively 10 per cent and 18.1 per cent during the first 15 days; the corresponding values in Litter II were 7.9 per cent and 15.9 per cent in the first 23 days.

When one considers that a prompt response to diet therapy is the prime desideratum in the treatment of hypoproteinemia, the value of adding hy-

drolyzed protein to an otherwise normal diet is rendered impressive by this result. It has already been shown by other workers that hydrolyzed protein alone seems not to have any special advantage over unhydrolyzed protein as the dietary source of nitrogen in the regeneration of blood serum protein.[1] It is evident from these experiments that the combination of both is singularly effective and can be utilized effectively in what Davis and Getzoff[6] term posthepatic and prehepatic hypoproteinemia of certain types.

When these results are considered in the light of the data in Table III, it becomes noteworthy that the increases in blood serum protein levels were achieved in both litters without significant increases in the total intake of nitrogen; i.e., the effects observed were obtained by a substitution of hydrolyzed protein for intact protein, not by addition to an otherwise adequate diet.

SUMMARY

1. Rats rendered hypoproteinemic by diet restriction were observed to recover from the protein depleted state by oral administration of a normal diet, and of a similar diet in which hydrolyzed protein was substituted for 10 per cent of the intact protein.

2. With practically equivalent total consumption on both diets, regeneration, as measured by increase in the blood serum protein level, was practically twice as rapid on the fortified diet during the first two to three weeks of the regeneration period.

The authors are indebted to R. A. Harte for assistance in the preparation of this manuscript.

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LABORATORY METHODS

GENERAL

IMPROVED METHOD OF TRANSPORTATION OF MATERIAL FROM GONOCOCCAL INFECTIONS*

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THE USE of gonococcus cultures has been considerably hindered by the lack of simple methods of transportation of gonococcal pus specimens to the laboratory. The method most used at present is to immerse a cotton swab containing the pus into broth or some other liquid medium in a carrying tube and to send it to the laboratory within six hours from the time the material was taken from the patient. When the culture material is in transit a longer time, negative culture results are unreliable.

Comparatively few investigators (Schofield,[1] Lumiere and Chevrotier,[2] Hauptman and Philadelphy,[3] Wortman, Gronau, Deakin, and Love,[4] Conn[5]) have studied the effect of time, temperature, and medium on the viability of gonococci in gonorrheal specimens. In the present study an attempt has been made to determine the influence of time and temperature on the survival of gonococci in specimens received on swabs immersed in broth, and to obtain data for the introduction of an improved transportation medium.

Material from known positive male and female cases of gonorrhea was used. The specimens reached the laboratory fifteen to thirty minutes after being taken. In the laboratory each swab was immediately streaked on plasma hemoglobin agar[6] and returned to the broth in the carrying tube. Two series of determinations were made; one series of 171 specimens in which the material was kept in broth at room temperature (22° C), and another of 162 specimens in which the specimens were kept at refrigerator temperature (6° C.). Following the inoculation of the immediate or control culture each specimen was plated on the same kind of medium every two hours for ten hours and again at twenty hours. The plates were incubated at 37° C. in an atmosphere of 8 to 10 per cent CO_2 for forty hours immediately after inoculation.

Table I shows the results obtained from examination of the two-hourly series of plates inoculated with known gonococcal material kept in broth at room temperatures (22° C.) and at refrigerator temperature (6° C.). The readings are separated into 4 plus (4+), 3 plus (3+), 2 plus (2+), 1 plus (1+), and plus-minus (±) according to the numbers of colonies found on the first set of streaked plates, which were the control plates of each series. In Table I, 3 plus and 1 plus readings were omitted since the results were similar to those

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TABLE I

NUMBER OF GONOCOCCUS COLONIES IN 66 FOUR PLUS SPECIMENS KEPT IN BROTH AT ROOM TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(4+) 200 COLONIES AND OVER	(3+) 100-200 COLONIES	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%						
After 2 hr.	97%	3%					
After 4 hr.	77%	19%	4%				
After 6 hr.	56%	21%	17%	6%			
After 8 hr.	40%	10%	30%	20%			
After 10 hr.	37%	13%	14%	20%	16%		
After 20 hr.	10%	0%	19%	25%	26%	10%	10%

NUMBER OF GONOCOCCUS COLONIES IN 36 TWO PLUS SPECIMENS KEPT IN BROTH AT ROOM TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE		
Incubated immediately	100%						
After 2 hr.	88%	12%					
After 4 hr.	36%	64%					
After 6 hr.	18%	75%	7%				
After 8 hr.	12%	44%	37%	0%	7%		
After 10 hr.	12%	6%	62%	13%	7%		
After 20 hr.	6%	8%	6%	64%	16%		

NUMBER OF GONOCOCCUS COLONIES IN 27 PLUS-MINUS SPECIMENS KEPT IN BROTH AT ROOM TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE				
Incubated immediately	100%						
After 2 hr.	100%						
After 4 hr.	96%	4%					
After 6 hr.	54%	37%	9%				
After 8 hr.	30%	50%	20%				
After 10 hr.	0%	76%	24%				
After 20 hr.							

NUMBER OF GONOCOCCUS COLONIES IN 50 FOUR PLUS SPECIMENS KEPT IN BROTH AT REFRIGERATOR TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(4+) 200 COLONIES AND OVER	(3+) 100-200 COLONIES	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE
Incubated immediately	100%						
After 2 hr.	100%						
After 4 hr.	58%	25%	17%				
After 6 hr.	39%	20%	21%	20%			
After 8 hr.	33%	11%	27%	29%			
After 10 hr.	27%	8%	17%	33%	15%		
After 20 hr.	5%	6%	17%	14%	34%	24%	0%

NUMBER OF GONOCOCCUS COLONIES IN 59 TWO PLUS SPECIMENS KEPT IN BROTH AT REFRIGERATOR TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(2+) 50-100 COLONIES	(+) 25-50 COLONIES	(±) 1-25 COLONIES	NO COLONIES	OVERGROWN PLATE		
Incubated immediately	100%						
After 2 hr.	100%						
After 4 hr.	60%	40%					
After 6 hr.	40%	53%	7%				
After 8 hr.	15%	59%	26%				
After 10 hr.	14%	36%	37%	13%			
After 20 hr.	7%	0%	46%	47%	0%		

TABLE I—CONT'D

NUMBER OF GONOCOCCUS COLONIES IN 22 PLUS-MINUS SPECIMENS KEPT IN BROTH AT REFRIGERATOR TEMPERATURE AND CULTURED AT 2 HOUR INTERVALS

	(±) 1-25 COLONIES	NO COLONIES	OVER-GROWN PLATE				
Incubated immediately	100%						
After 2 hr.	100%						
After 4 hr.	82%	18%					
After 6 hr.	73%	27%					
After 8 hr.	42%	58%					
After 10 hr.	12%	88%					
After 20 hr.	0%	100%	0%				

obtained from 4 plus and plus-minus cultures respectively. In considering the results of Table I it is essential to take into account the probable error in the percentages due to the mechanical factors involved in streaking a number of plates with one swab.

Out of a total of 171 positive specimens kept in broth at room temperature for twenty hours, 95, or 52 per cent, failed to show gonococci on culture. Of the 162 refrigerator specimens, 82, or 50 per cent, failed to show gonococci on culture.

The tests just described indicate that there is a diminution in the number of gonococci in storage of the specimen in broth, and that the diminution is in ratio to the length of time in storage. When specimens are kept in broth at room temperature, the culture frequently becomes overgrown with contaminating bacteria which prevent the isolation of the gonococci. Therefore when broth is used as a transportation medium, it is necessary to reduce the time in transportation to a maximum of four hours, particularly in doubtful cases.

In a subsequent study, in which plasma hemoglobin agar plates instead of broth were used for transportation, the following method was adhered to: A set of three plates was used for each known positive case. The plates were inoculated in the clinic and sent to the laboratory without delay. One plate from each specimen was incubated immediately at 37° C. for forty hours, in an atmosphere of 8 to 10 per cent CO_2. The second plate was kept at room temperature (22° C.) for twenty to twenty-four hours, and the third plate was placed in the refrigerator (6° C.) for twenty hours. At the end of twenty to twenty-four hours the room and refrigerator plates were incubated in the same manner as the immediate plate. The results obtained from the immediate and delayed cultures are shown in Fig. 1.

By transporting material on plasma hemoglobin agar, the loss of positives at room temperature after twenty to twenty-four hours was reduced from 52 per cent loss in broth to 11 per cent on agar, and at refrigerator temperature, from 50 per cent loss in broth to 14 per cent on agar.

Although transportation on solid medium is an improvement over the broth method, the loss of 11 to 14 per cent of positives in twenty to twenty-four hours is too great. Various substances were therefore incorporated in the medium in an attempt to find one which would effect better survival of the gonococci during transportation. When cysteine monohydrochloride, which had been used by

Cohn[5] in liquid media, was added to plasma hemoglobin agar to a concentration of 0.05 to 0.03 per cent, the gonococci in the specimen on these plates remained alive in practically every instance, when stored at room temperature for twenty to twenty-four hours before incubation.

In the preparation of the cysteine medium, a sterile 10 per cent aqueous solution of cysteine monohydrochloride is added to the melted proteose peptone agar after it has been cooled to 55° C., and before phosphate, plasma, and hemoglobin are added. This precaution is taken to avoid the formation of a precipitate. Cysteine monohydrochloride is added to a concentration of 0.03 per cent. Fig. 2

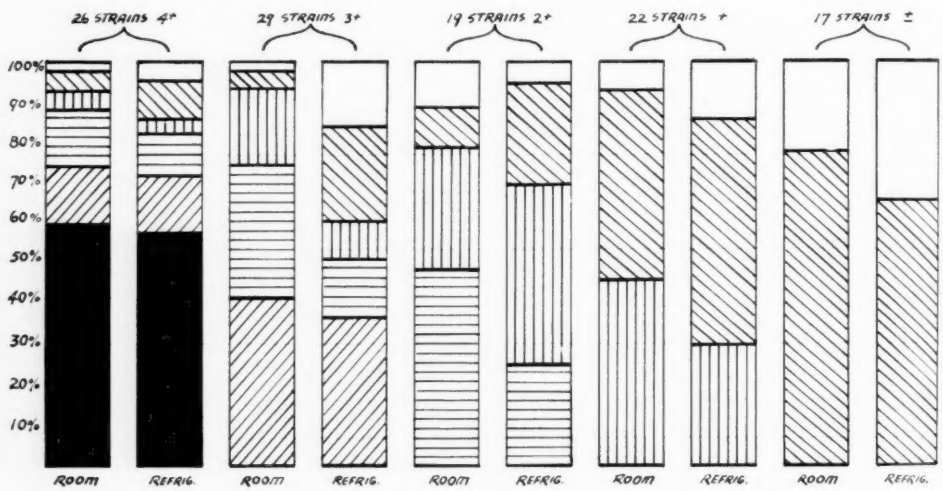

Fig. 1.—One hundred thirteen known positive strains from males streaked on horse plasma hemoglobin proteose peptone agar and left at room and refrigerator temperature for 24 hours before incubation.

Eleven strains lost at room temperature at end of 24 hours; 15 strains lost at refrigerator temperature at end of 24 hours.

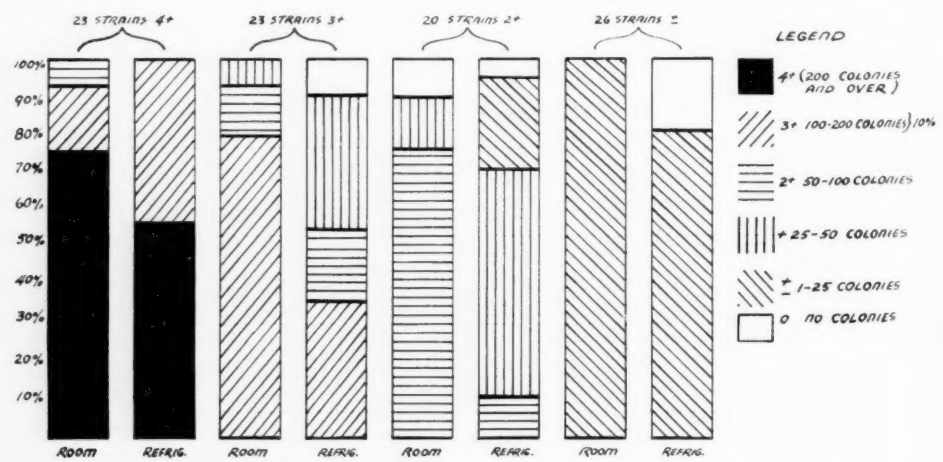

Fig. 2.—Ninety-two known positive strains from males streaked on 0.03 per cent cysteine monohydrochloride horse plasma hemoglobin agar and left at room and refrigerator temperature for 24 hours before incubation.

Eight strains lost at refrigerator temperature in 24 hours; none lost at room temperature.

shows the results on 92 known positive specimens from males, streaked on 0.03 per cent cysteine agar plates, and kept at room or refrigerator temperature for twenty to twenty-four hours. In this series no positives were lost at room temperature, and 8.7 per cent were lost at refrigerator temperature.

During this experiment l-cystine was also tested (Higginbotham[7]). This chemical inhibited the growth of gonococci when 0.1 per cent, 0.05 per cent, or 0.025 per cent was added to plasma hemoglobin agar. When 0.1 per cent was added to plasma hemoglobin agar containing veal or beef infusion as a base, it produced a luxuriant growth of gonococci. It seems that l-cystine requires a meat infusion base in order to stimulate good growth of gonococci. Because a meat infusion base is required and because the chemical dissolves only in the autoclave, l-cystine was not tested in transportation media.

The cysteine plasma hemoglobin agar was then tested as a transportation medium for general routine cultures. Each of 1,250 specimens were streaked directly on three plates, two of which contained plasma hemoglobin agar, and one, plasma hemoglobin agar with 0.03 per cent cysteine monohydrochloride. The plates without cysteine were incubated immediately as controls. The cysteine plate was kept at room temperature for twenty to twenty-four hours and then incubated in the usual manner. There were 240 positive cases, of which six control cultures were negative when the corresponding cysteine plates were positive, and five cysteine cultures negative when the corresponding control plates were positive. There was a drop in the number of colonies on cysteine plates in about 25 per cent of the positive cultures. This drop in number of colonies varies and is not in proportion to the number of colonies on the control plates. Almost all of the plus-minus cultures remained positive. The exposure of the streaked plates to out of doors winter temperatures for two to three hours did not affect the culture results.

SUMMARY

1. The streaking of gonococcal pus directly on to enriched solid medium containing 0.05 to 0.03 per cent of cysteine monohydrochloride saves 100 per cent of the positives when at room temperature up to ten hours, and about 98 per cent when left at room temperature from twenty to twenty-four hours.

2. In specimens kept in broth at 22° C. for six hours 10 per cent of positives are lost, and for ten hours 26 per cent of positives are lost. On plasma hemoglobin agar none are lost up to ten hours. When specimens are kept twenty hours at 22° C., 52 per cent of positives are lost in broth, 11 per cent on plasma hemoglobin agar, and only about 2 per cent on plasma hemoglobin agar with cysteine.

3. Transportation on solid medium is safer at 22° C. than at 6° C. Some cultures (8.7 per cent) are destroyed at refrigerator temperature when exposed to it longer than ten hours.

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AN ELECTRICAL PROCEDURE FOR THE REPURIFICATION-CONCENTRATION OF ANTITOXIN*

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WHEN antitoxin ages, the antibody which it contains loses titer; this in all probability is a result of protein transformations which cannot be stopped. When a large decrease in antibody content occurs, the material becomes too weak for therapeutic use. Rather than discard this valuable material, it is desirable to increase its potency again by reducing its volume. The amount of total nitrogen possible per unit volume, however, is fixed by law, and therefore this reduction in bulk must be accompanied by a corresponding elimination of immunologically inert protein.

The salting-out method utilizing such salts as sodium and ammonium sulphate, first developed by Gibson,[1] and then improved upon by a host of others, is the most common method used today for purifying and increasing the titer of antitoxic plasma. Experience has shown that when this procedure is again employed in an attempt to repurify deteriorated antitoxin preparations, the losses sustained in active material are large and the increase in potency is small. In view of the fact that the euglobulin, albumin, and fibrinogen fractions had been eliminated by the first purification of the antiserum, then, in the scope of this method, there is no more immunologically inert protein to be eliminated by repurification. Consequently an increase of antibody pseudoglobulin per unit volume is impossible.

An electrical process is described here which treats the antibody protein molecular aggregates in such a manner as to allow a further purification with a subsequent increase in antitoxin value per milligram of nitrogen. Furthermore, the color-carrying protein, which had been oxidized and reduced to colors varying from light brown to red, is to a great extent eliminated with the inert fraction, leaving the reprocessed material lighter in color.

The degree of increase in potency per unit of nitrogen has ranged with this process from 15 to 80 per cent. The variation is caused by the condition of the

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different lots reprocessed. Those which were old and whose protein content had become more stable did not allow as good a reconcentration as more recent material where protein transformations were more readily induced. The losses in antitoxin sustained by this process vary from less than 5 to more than 20 per cent depending again upon the condition of the material and upon the degree of reconcentration which one tries to attain.

The data in Table I give the variations in losses and in potency increase with six different lots of diphtheria antitoxin.

TABLE I

PREPARATION	ORIGINAL POTENCY IN GUINEA PIG UNITS AT 20% TOTAL SOLIDS (30.4 MG. N/GM.)	FINAL POTENCY IN GUINEA PIG UNITS AT 20% TOTAL SOLIDS (30.4 MG. N/GM.)	% LOSS IN TOTAL UNITAGE	% POTENCY INCREASE
1	3,540	4,580	6.6	29.3
2	3,540	5,420	4.1	53.1
3	1,190	1,840	0	54.6
4	1,630	1,970	0	20.8
5	1,120	1,740	28.7	55.3
6	1,137	1,940	0	70.6

The apparatus and procedure of the method are described here in detail because it has been found that even small variations in size of tubes, cellophane, amount of water added to the units, pH values, etc., make a decided difference in the quality of the final product.

APPARATUS NO. 4

The metal tubes are five inches long, one inch in diameter, and one thirty-second of an inch thick. They are made in the form of long, watertight thimbles. Zinc has been found to be the most efficient metal to use, because, for one reason, its ionization does not cause the serum to undergo rapid oxidation-reducing reactions accompanied by color formation.

The tubes are filled with tap water and the platinum electrodes are inserted as shown in Fig. 1.

There are two semipermeable membranes made of water-softened cellophane (Dupont, No. 600) and each contains three tube electrodes. Thus, there are three anode tubes in one membrane and three cathode tubes in the other.

The membranes are filled with tap water equal to the volume of antitoxin to be processed. The tube electrodes are immersed therein. For the support of these different units, the author has arranged a flat piece of glass with suitable holes to fit over the container, so that a metal frame firmly attached to each cellophane bag can be screwed onto it. Other holes allow the tube electrodes to be immersed in the fluid of each membrane.

The current used has been 120 volts direct current with the circuit as shown in Fig. 1. The current from a battery in conjunction with suitable resistance can be used instead, if desired. Three variable resistances are used instead of a large equivalent one for purposes of convenience only.

From 30 to 150 milliamperes are passed through the antiserum with an electromotive force of from 30 to 120 volts. This current, when used with this apparatus, has been found to cause no antibody destruction.

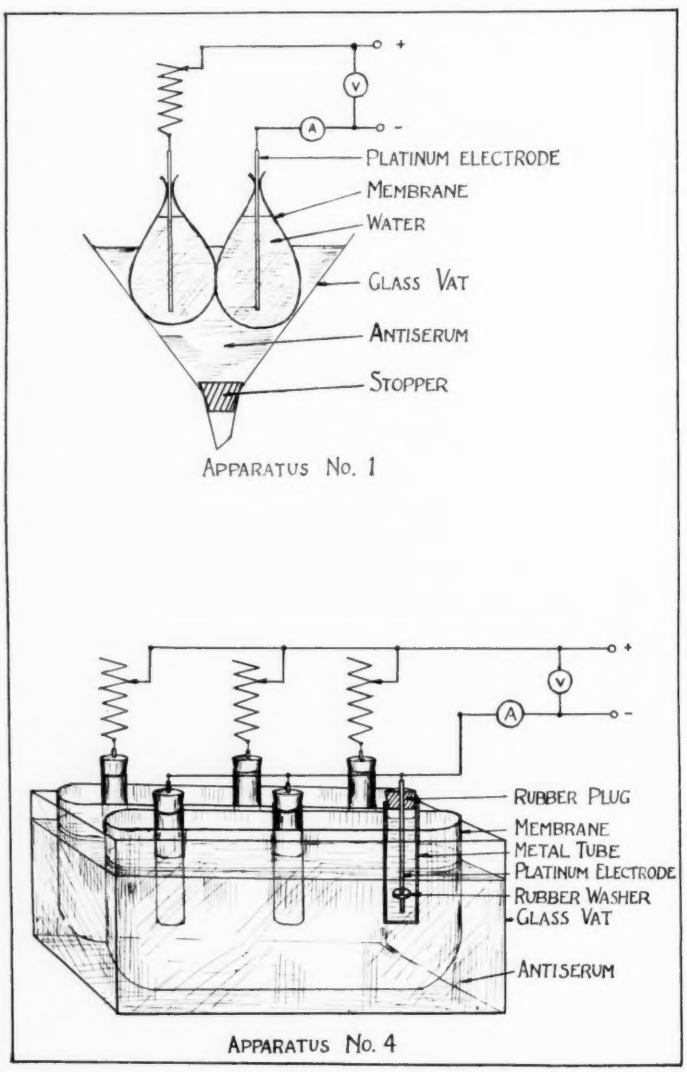

Fig. 1.

PROCEDURE

All hydrogen ion values referred to in the following description have been determined by the color comparator method. Gamma dinitrophenol and para and meta nitrophenol indicators with appropriate disc standards were used. This method has been checked with the potentiometric method and it has been found to be sufficiently accurate. It has been employed in this work because it lends itself more readily to the running of many determinations in a short time.

The antitoxin material to be processed is adjusted to a hydrogen ion value of pH 5.1 with normal acid acetic. It is dialyzed against running water until all the salts possible have been eliminated and the hydrogen ion value has increased to between pH 5.9 and pH 6.5. It is then diluted with distilled water to a nitrogen content of between 8 and 12 mg. of nitrogen per gram of material. The hydrogen ion value is again adjusted to pH 5.1 as above.

Thus prepared, the material is poured into the glass container of the apparatus, the membrane units are immersed therein, and the electric current is turned on.

When a preparation which permits visibility through it is being processed, one can immediately see stringlike oozing and settling of colloidal material all around and under the cathode membrane unit.

The material should be stirred from time to time manually unless a slow mechanical stirrer is used.

The hydrogen ion concentration must be checked often. It will be noted that as the process continues, more and more protein matter precipitates in the region of the cathode unit, accompanied by a slow but constant rise in pH value. When pH 5.9 has been attained in the mixture, the process is stopped.

It has been determined by many tests for antitoxin that the protein precipitated up to this point is mostly euglobulin. This fraction of protein is one of the immunologically inert globulin components which is precipitated between 0 and 30 per cent saturation with ammonium sulphate and whose existence has been further proved by the analysis of serum[3] with the Tiselius electrophoretic apparatus[4, 5] and the ultracentrifugal sedimentation method of Svedberg.[6] It contains no antitoxin except for a little which has been adsorbed by the precipitating proteins. This precipitate is separated from the mother liquor by filtration or centrifuging, and the precipitate is washed free of the adsorbed antitoxin by mixing it well with distilled water. The solid is sedimented or filtered again and is discarded. The washings are pooled with the filtrate or supernatant of the first separation.

In this manner the antitoxin has been repurified of an immunologically inert fraction of protein which, for the most part, would not have been present in the scope of the salting-out procedure.

The immunologically useful protein, namely, the pseudoglobulin, which remains in the clear purified material, can now be brought to a desired smaller volume by employing the following technique:

Normal sodium hydroxide is added slowly and with constant stirring to the purified colloidal solution. As the hydrogen ion concentration is reduced, more and more of the protein content precipitates out as in isoelectric precipitation. Finally at pH 7.0 to pH 7.4 all of the useful protein should be in the solid state. The precipitate is collected by filtration and the filtrate is discarded.

Normal acid acetic* is added slowly to the solid, with constant mixing, until the precipitate is in the colloidal state. The pH value of the resulting solution will be between pH 5.6 and pH 5.8. Solid sodium chloride to 1 per cent is added and the material is dialyzed against running water until only a trace of chloride

*An alkali has the same effect; however it has been found that antitoxin antibody is more tolerant to acidity than to alkalinity.

ion remains. One per cent solid sodium chloride and a suitable preservative are added after dialysis. The hydrogen ion value is set at pH 7.2 and the material is now ready for sterilization and standardization.

DISCUSSION

If according to Kruyt,[2] colloidal particles of the emulsoid type possess two factors which tend to make them stable with respect to precipitation, namely charge and hydration, and that either of these is enough to prevent aggregation of particles and flocculation of the colloid, then the apparatus as used satisfies both of these conditions and causes selective precipitation of the protein content of immune serum.

To develop the present equipment Apparatus 1 was first employed. Material processed with it showed oily, colloidal oozing and settling around and under the anode or cathode units depending on the direction of electrophoresis caused by the pH value of the antiserum.

When the process is begun with this apparatus at a neutral pH value, the settling of material at the anode unit is followed by the formation of a zone of clear fluid at the top of the serum in this region. The pH value of this clear fluid is acid and presently a hazy, ill-defined precipitate of pseudoglobulin separates out and slowly settles to the bottom layer. The clear zone then loses definition and assumes the pH value and the color of the bulk. There is no definite lasting precipitate, however, and the reaction of the serum during the process slowly becomes acid. This apparatus, therefore, although it causes protein transformations, is not enough of itself to cause aggregation of molecular particles and flocculation.

To add the necessary factor the metal jackets were added to the units, and their slow ionization completes the mechanism of the desired reaction. When antiserum at a neutral pH value is processed with this apparatus, the proteins precipitate in a thick coat on the outside surface of the anode membrane unit. When the process is begun with the serum at pH 5.1, however, the desired selective precipitation is obtained.

The hydrogen ion value of the fluids in the anode and cathode membrane units at the end of the process show the former to be between pH 6.2 and pH 6.5 while that of the latter is above pH 8.4. It has been found that an alkaline compound of the metal is formed in these units. The alkalinity produced in the anode unit, however, is transmitted to the serum as fast as it is formed by electrophoretic action. The result is a gradual rise in the pH value of the serum while that of the unit fluid itself remains constant. The cathode unit does not transmit any ion to the serum and therefore the reaction of this fluid becomes alkaline.

To demonstrate these points an alkali was added to the fluid of the anode unit, with or without metal jackets, and the pH value of the serum rose immediately. The reverse was true when an acid was added. When either acid or alkali were added to the fluid of the cathode unit no change in the pH value of the serum occurred.

That the anode metal jacket is the real functional one was shown by processing some material with a platinum electrode in the cathode unit and the

regular metal jacket in the anode unit. The serum underwent selective precipitation as usual, but the degree of reconcentration was not as good as when the complete apparatus was used. No selective precipitation occurred when the metal jacket was put in the cathode unit and the platinum electrode in the anode.

The anode tube is gradually used up during the process by the slow ionization of the metal while the outside surface of the cathode tube shows a black region, which is caused by a deposition of metal ion from the system as in electroplating.

The apparatus also causes some electrodialysis of the serum to occur making possible protein precipitation with very small quantities of metal ion.

SUMMARY

An electrical process is described which makes possible the repurification of deteriorated antitoxin material.

The apparatus is planned in a manner that allows purification and final reduction of volume by isoelectric precipitation of the proteins, thus eliminating the use of saturated salts.

The procedure is simple and it can be adapted to large-scale work.

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A STUDY OF MEDIA FOR TYPHOID BLOOD CULTURES*

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INTRODUCTION

THE isolation and identification of *Eberthella typhosa* from the blood, feces, or urine of a patient has long been recognized as the most valuable laboratory aid in the diagnosis of typhoid fever. While extensive experimentation has yielded a number of very productive types of media for the isolation of *E. typhosa* from the feces and urine, relatively little choice of media is available for the isolation of this organism from the patient's blood. The glycerol-bile-peptone medium devised by Conradi[1] in 1906 is probably the most frequently used medium for typhoid blood cultures, particularly clot cultures, at the present time. It is commonly believed, as stated by Coleman,[2] that the bile in this medium dissolves the blood clot, but in the writer's experience little if any dissolution of the clot occurs during the usual incubation period of seven to ten days at 37° C. The popular belief in this property of bile is probably based upon the misinterpretation of Conradi's original statement that the bile *prevents the formation* of a clot when whole blood is inoculated into the medium.

The routine use of clot cultures for the diagnosis of typhoid fever has become a common practice in public health laboratories during recent years, since both an agglutination test and a blood culture can be performed on the same specimen. While this practice is usually satisfactory, there is apparently an unavoidable tendency for many of the clots to become contaminated either in the taking of the blood or during transportation to the laboratory. The writer is aware of public health laboratories where as high as 50 per cent of the clots have been contaminated, especially during the warm months, despite meticulous aseptic technique after the specimens reached the laboratory. Since the majority of contaminating organisms from clot cultures in Conradi's medium are staphylococci, it would appear desirable to add some substance to the medium which would suppress the growth of staphylococci without inhibiting the multiplication of the typhoid bacillus, if present. Recently Hager and Grubb[3] reported that p-bromocinnamic acid would exhibit such a selective inhibitory action against staphylococci without affecting the growth of *E. typhosa*. Since this preliminary study suggested the utility of p-bromocinnamic acid for typhoid clot culture media, an extensive investigation was made to determine the practicability of adding this acid to Conradi's medium. The present report summarizes the results of this study. In addition, a comparative study was made with media containing sodium desoxycholate to determine whether or not this agent, suggested by Sellers[4] for blood cultures, was more or less effective than p-bromocinnamic acid in selectively inhibiting the growth of staphylococci.

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EXPERIMENTAL

Preliminary experiments indicated that the selective inhibitory action of p-bromocinnamic acid for staphylococci was dependent upon the concentration of the acid and the pH of Conradi's medium to which it had been added. It was found that the lower the pH of the medium, the less the amount of p-bromocinnamic acid required to suppress the growth of staphylococci. Likewise, if sufficient p-bromocinnamic acid were added at a low enough pH, a point was reached where the growth of the typhoid bacillus as well as that of the staphylococci was completely inhibited. It was therefore obvious that the proper balance of pH and concentration of p-bromocinnamic acid must be found which would inhibit the growth of staphylococci without affecting the growth of *E. typhosa*. After several trials it was found that if Conradi's medium was adjusted to pH 5.0 with p-bromocinnamic acid in a concentration of 1:5000, the medium would exhibit the desired selectivity. Table I indicates the results obtained with this combination containing 10 per cent of defibrinated rabbit's blood. Freshly isolated strains of *E. typhosa** and *Staphylococcus albus* were inoculated into the medium and plate counts in nutrient agar made immediately after inoculation and after twenty-four hours' incubation at 37° C.

It is evident from the data shown in Table I that there is a definite bactericidal action on both strains of staphylococci, while the multiplication of the typhoid bacillus was apparently unaffected. It is to be noted that an extremely large inoculum was used in the above experiment.

TABLE I

SELECTIVE BACTERICIDAL ACTION OF P-BROMOCINNAMIC ACID (1:5000) IN CONRADI'S MEDIUM (PH 5.0) CONTAINING 10 PER CENT BLOOD

ORGANISM	TOTAL PLATE COUNT PER C.C. OF MEDIUM	
	IMMEDIATELY AFTER INOCULATION	24 HOURS' INCUBATION AT 37° C.
E. typhosa No. 1	940,000	51,000,000
E. typhosa No. 2	400,000	34,000,000
S. albus No. 1	21,000	40
S. albus No. 2	100,000	23,000
Control Medium Without P.Bromocinnamic Acid		
E. typhosa No. 1	1,060,000	20,000,000
E. typhosa No. 2	920,000	22,000,600
S. albus No. 1	570,000	46,000,000
S. albus No. 2	860,000	64,000,000

In order to determine the effect of various concentrations of blood upon the inhibitory action of p-bromocinnamic acid, defibrinated rabbit's blood was added to Conradi's medium in amounts varying from 1 to 30 per cent. When the concentration of blood exceeded 15 per cent, there was a definite reduction in the inhibitory activity of p-bromocinnamic acid, which became more pronounced in the higher concentrations of blood. It is difficult to estimate how much blood or serum is liberated in the usual clot culture, but for the purposes of experi-

*Freshly isolated strains of *E. typhosa* from blood cultures were obtained through the courtesy of Dr. C. A. Perry, Director, Bureau of Bacteriology, Maryland State Department of Health.

mentation a concentration of 10 per cent has been selected as representing an average amount and has been employed throughout the experiments reported here.

While a concentration of 1:5000 p-bromocinnamic acid apparently had no inhibitory effect upon the growth of *E. typhosa* when a very large inoculum was used (400,000 to 940,000 per c.c. as shown in Table I), it was deemed advisable to determine the effect of this concentration upon a small inoculum. The number of organisms which should be used as an inoculum to simulate the number found in the blood stream of a typhoid patient is probably very small. Müller and Gröff[5] made plate counts from blood cultures of 256 typhoid cases and found that 82.2 per cent of the cultures contained between 1 and 50 organisms per c.c. of blood. Plate counts made on the blood of 43 patients in the University of Maryland Hospital showed between 1 and 63 typhoid bacilli per c.c. of blood; however, when a number of hours intervenes between the taking of the blood and the laboratory examination, such as occurs when specimens are sent through the mail to public health laboratories, it is difficult to predict what degree of multiplication will take place. For experimental purposes, inocula containing from 10 to 20 organisms were employed, and this small number of typhoid bacilli was markedly inhibited in Conradi's medium adjusted to pH 5.2 and containing 1:5000 p-bromocinnamic acid.

Since Sellers[4] had reported the use of desoxycholate broth for the culture of clots from suspected cases of typhoid fever, it was considered of interest to determine how this medium compared with the p-bromocinnamic acid medium described above. The formula for this medium, kindly supplied by Harris,[6] is as follows: peptone, 1 per cent; Na_2HPO_4, 0.35 per cent; sodium desoxycholate, 0.1 per cent. The hydrogen-ion concentration of the medium is adjusted to pH 7.0 to 7.2 before autoclaving at 15 pounds' pressure for fifteen minutes. Small inocula (10 to 25 organisms) of two recently isolated strains of staphylococci and *E. typhosa* were inoculated into the p-bromocinnamic acid and the desoxycholate media, and incubated 24 hours at 37° C. when plate counts in nutrient agar were made. The results of this experiment, which was repeated several times, are given in Table II.

It is evident from the data in Table II that while p-bromocinnamic acid is bactericidal for staphylococci, it is also markedly inhibitory for typhoid bacilli when a small inoculum is employed. On the other hand, the desoxycholate broth completely suppressed the growth of staphylococci while allowing considerable growth of *E. typhosa*. These data indicate clearly that sodium desoxycholate is a much more effective substance than p-bromocinnamic acid for the selective inhibitory action on staphylococci in typhoid blood cultures.

To determine what effect variations in the pH of the medium and concentration of sodium desoxycholate would have upon the selective inhibitory action of the desoxycholate broth, various portions were prepared containing from 0.05 per cent to 0.3 per cent sodium desoxycholate with the hydrogen-ion concentration of the broth ranging from pH 6.0 to 8.0. These portions, containing 10 per cent defibrinated rabbit's blood, were inoculated with approximately 10 staphylococci or typhoid bacilli per 5 c.c. of medium and incubated

twenty-four hours at 37° C. Each portion was then plated in nutrient agar to determine the total number of organisms present. The results of this experiment are shown in Table III.

TABLE II

COMPARISON OF THE BACTERICIDAL ACTION OF P-BROMOCINNAMIC ACID IN CONRADI'S MEDIUM WITH THAT OF DESOXYCHOLATE BROTH

MEDIUM	TOTAL PLATE COUNT PER C.C. OF MEDIUM AFTER 24 HOURS' INCUBATION AT 37° C.			
	ORGANISM		ORGANISM	
p-bromocinnamic acid (1:5000)	*S. albus* No. 1	0	*E. typhosa* No. 1	73
	S. albus No. 2	0	*E. typhosa* No. 2	1,070
Sodium desoxycholate (1:1000)	*S. albus* No. 1	0	*E. typhosa* No. 1	500,000
	S. albus No. 2	0	*E. typhosa* No. 2	700,000

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM DESOXYCHOLATE AND PH OF THE MEDIUM ON THE GROWTH OF *E. Typhosa* AND *S. Albus* IN DESOXYCHOLATE BROTH

ORGANISM	pH	TOTAL PLATE COUNT PER C.C. OF MEDIUM AFTER 24 HOURS' INCUBATION AT 37° C.			
		0.05%*	0.1%	0.2%	0.3%
S. albus No. 1	6.0	0	0	0	0
	7.0	0	0	0	0
	8.0	0	0	0	0
E. typhosa No. 1	6.0	22,100,000	48,000,000	25,000,000	28,000,000
	7.0	30,000,000	69,000,000	35,000,000	71,000,000
	8.0	11,000,000	24,400,000	71,000,000	34,000,000

*Concentration of sodium desoxycholate.

The data in Table III show the complete inhibition of growth of staphylococci while *E. typhosa* grew luxuriantly. Nutrient broth control tubes (pH 7.0) containing 10 per cent blood inoculated at the same time and incubated in a similar manner contained 153,000,000 staphylococci and 130,000,000 typhoid bacilli per c.c. of the culture. Thus while the sodium desoxycholate in the concentrations employed in this experiment has some inhibitory action on *E. typhosa*, it is probably insignificant so far as the practical usefulness of the medium is concerned. While the number of typhoid bacilli developing in the various concentrations of sodium desoxycholate at different pH values varied from 11,000,000 to 71,000,000 per c.c., there is no apparent relationship between the two variables and the number of organisms developing. It is believed that for practical purposes the desoxycholate broth will exhibit adequate selective inhibitory action in any of the combinations of pH and in the concentration of sodium desoxycholate shown in Table III. The fact that neither the concentration of the sodium desoxycholate nor the pH of the medium requires critical adjustment increases the practical usefulness of the medium.

Since it was found that the inhibitory action of p-bromocinnamic acid on staphylococci was nullified to a considerable extent by concentrations of blood exceeding 15 per cent, it was considered of practical importance to determine whether or not blood had a similar effect in the desoxycholate medium. Defibrinated rabbit's blood in concentrations ranging from 1 to 30 per cent was added

to desoxycholate broth containing 0.1 per cent sodium desoxycholate and adjusted to pH 7.0. Small inocula (100 to 200 organisms) of *E. typhosa* and *S. albus* were inoculated into the broth containing various amounts of blood and incubated at 37° C. for twenty-four hours when plate counts in nutrient agar were made. Even in the media containing 30 per cent blood there was no reduction in the inhibitory action of sodium desoxycholate on staphylococci, while in the media containing only 1 per cent blood, there was no inhibitory action on the growth of typhoid bacilli. This experiment indicates that variations in the concentration of blood, within the probable limits found under practical conditions, do not affect the selective inhibitory action of sodium desoxycholate in the medium described.

While sodium desoxycholate was shown to display an effective bactericidal action on staphylococci in previously described experiments where small inocula were used, it appeared advisable to determine the inhibitory action of this substance against a large inoculum, since considerable multiplication of contaminating staphylococci may occur when the blood specimen is sent through the mail to the diagnostic laboratory. In order to study this problem, 0.1 per cent desoxycholate broth adjusted to pH 7.0 and containing 10 per cent blood was prepared. Varying numbers of staphylococci were inoculated into tubes containing 5 c.c. of the medium and incubated twenty-four hours at 37° C. when plate counts were made to determine the number of surviving organisms. The results of this experiment are shown in Table IV.

TABLE IV

ACTION OF DESOXYCHOLATE BROTH UPON VARIOUS NUMBERS OF *Staphylococcus albus*

NUMBER OF ORGANISMS INOCULATED INTO 5 C.C. OF MEDIA	NUMBER OF ORGANISMS VIABLE AFTER 24 HOURS' INCUBATION AT 37° C.
12,800,000	4,000
1,280,000	3,050
128,000	450
12,800	50
1,280	0
128	0

This experiment demonstrated that sodium desoxycholate has a very effective bactericidal action against large numbers of staphylococci; and even though some of these organisms do survive over a 24-hour period, the number of surviving organisms is insignificant compared to the number of typhoid bacilli which grow during this period under the same conditions (see Table III).

DISCUSSION

The experiments described in this report indicate that while p-bromocinnamic acid shows a selective inhibitory action against staphylococci when the concentration of the acid and the pH of the medium are properly adjusted, this selective activity probably cannot be utilized in a medium for the culture of blood clots because small numbers of typhoid bacilli are inhibited. On the other hand, a simple broth containing from 0.05 to 0.3 per cent sodium desoxycholate and adjusted to pH 6.0 to 8.0, shows a remarkably clear-cut bactericidal

action against staphylococci without a significant degree of inhibition for the typhoid bacillus. This selective activity is manifest despite wide variations in the amount of blood present and despite an enormous inoculum of staphylococci. These experiments suggest that a desoxycholate broth, similar to that described, should prove very helpful to laboratories where difficulty is experienced in avoiding or eliminating contamination of blood clot cultures with staphylococci.

SUMMARY

1. A study was made of the utility of p-bromocinnamic acid incorporated in Conradi's medium for the purpose of inhibiting the growth of contaminating staphylococci. Although p-bromocinnamic acid showed a selective inhibitory action against staphylococci, it also inhibited small numbers of typhoid bacilli and therefore was not considered of practical value for the culture of blood clots from suspected cases of typhoid fever.

2. A simple broth containing sodium desoxycholate displayed an effective inhibitory action against staphylococci without an appreciable bacteriostatic effect upon the typhoid bacillus. The broth does not require critical adjustment of pH or concentration of sodium desoxycholate, and is effective in various concentrations of blood against large numbers of staphylococci.

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MICROSCOPIC OBSERVATION OF COLLODION PARTICLES AS INDICATORS OF TYPE-SPECIFIC PNEUMOCOCCIC IMMUNE REACTIONS

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THE object of this study was to detect the presence of minute amounts of pneumococcal soluble-specific substances (S.S.S.) in vitro. A sensitive microscopic technique was developed which was compared with a slightly modified method of Goodner's[1] and with the ordinary precipitation tests.

In 1932 Freund[2] demonstrated that anthrax soluble carbohydrate hapten was adsorbed by collodion particles; for on adding anthrax-immune serum to the hapten-sensitized particles, agglutination resulted. The agglutination was observed best in a hanging-drop preparation, but the titers of the reagents were not reported.

Experimental.—Collodion particles were prepared according to the method of Cannon and Marshall[3] except that double-distilled rather than triple-distilled water was used, as recommended by Eisler.[4] The particles obtained from 75 ml. of 5 per cent acetone stock solution, after aspirating, washing, and centrifuging, were suspended in 100 ml. of double-distilled water and used without further diluting.

Into agglutination tubes was added 0.02 ml. of collodion suspension followed by 0.1 ml. of S.S.S. dilution. The latter was made up in physiologic saline. After thorough mixing, the tubes were allowed to stand for from five to ten minutes at room temperature, then 0.1 ml. of antiserum added. The contents were mixed again, capped with rubber stoppers, and placed in the refrigerator overnight. With each test, a serum control (0.02 ml. collodion suspension plus 0.1 ml. antiserum) and an S.S.S. control (0.02 ml. collodion suspension plus 0.1 ml. S.S.S. dilution) were made. Another set of tubes containing the same amounts of reagents were simultaneously made up for the so-called "collodion-fixation test" of Goodner.[1] A third set without collodion particles constituted the ordinary precipitation test.

Microscopic Technique.—A drop of the collodion plus antigen (S.S.S.) plus antiserum suspension is placed on a plain glass slide. Next to it is placed a drop of the serum plus collodion control and next to this, a drop of the antigen plus collodion control. Each drop is covered with a coverglass and examined under the oil immersion lens. It is desirable to flip each tube gently about ten times just before withdrawing the drop of suspension. In the mounts containing the higher concentrations of S.S.S., one sees definite amorphous masses containing enmeshed collodion particles. These are designated 4 plus and 3 plus in Table I. A 2 plus reading signifies the presence of relatively large clumps

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of collodion particles without a trace of the amorphous precipitate. A plus reading means that the majority of the fields examined contain more clumps (small in size) and fewer single particles as compared with the controls. With one exception, all the serum and antigen controls thus far examined contained some small collodion clumps and many single particles; that is, the dispersion was good. The exception, a concentrated horse serum plus collodion control, contained large clumps of particles and was similar to a 2 plus reading.

The results of a typical comparative test are shown in Table I.

TABLE I

TITRATION OF S.S.S. III AGAINST A RABBIT TYPE III ANTIPNEUMOCOCCUS SERUM (UNDILUTED): COMPARATIVE TESTS

TUBE	DILUTION OF S.S.S.III 1:	MICROSCOPIC READING	MACROSCOPIC READING	PRECIPITATIVE TEST WITHOUT COLLODION
1	5×10^{-5}	4+	3+*	2+
2	10^{-6}	4+	2+†	+
3	3×10^{-6}	4+	+	±
4	5×10^{-6}	3+	±	–
5	5×10^{-7}	2+	–	–
6	10^{-9}	+	–	–
Serum control		–	–	–
S.S.S. controls	5×10^{-5}	–	–	–
	5×10^{-6}	–	–	–

*Disc.

†Coarse particles, not easily broken up on vigorous flipping.

SUMMARY

A microscopic method of observing collodion particle agglutination is described. In higher dilutions of pneumococcus S.S.S., the method gives positive results, while negative results are obtained by macroscopic examination either of the same tests or of ordinary precipitation tests.

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CHEMICAL

A METHOD AND APPARATUS FOR SHELL FREEZING AND RAPID DRYING OF PLASMA AND OTHER PRODUCTS FROM THE FROZEN STATE BY LOW TEMPERATURE WATER VAPOR CONDENSATION IN VACUO*

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INTRODUCTION

HUMAN plasma transfusion is now generally accepted as an important therapeutic agent in the treatment of shock, associated with trauma, hemorrhage, or burns as well as of hypoproteinemias, hypoprothrombinemias, and of some hemorrhagic conditions. Preservation of plasma in the frozen state has been recommended as the safest, most efficient, and practical method for the average hospital.[1] The establishment of "plasma banks" in which the plasma is maintained frozen will allow most institutions to meet the routine or emergency needs of the communities which they serve in almost all instances. This is true of the present national emergency. "Banks" of frozen plasma are being established in many hospitals throughout the country in view of the increasing danger of industrial accidents associated with large scale production and the possibility of enemy action within the United States. Large stores of dried plasma are being prepared for use with the Armed Forces beyond the limits of the continental United States. Conditions do exist, and further situations may arise, in which a certain amount of dried plasma is needed for civilian use.

Very few hospitals at present are in a position to offer the use of dried plasma for their patients, except through outright purchase of the material. This situation in effect denies the patient the opportunity of availing himself of the use of friends and relatives as volunteer donors. In a previous publication[1b] it has been pointed out that this difficulty could be overcome if a group of hospitals would cooperate in the preparation of dried plasma. Since the preservation of plasma in the dried state is only seldom essential in the ordinary civilian practice, a single large institution could easily dry a sufficient quantity to meet the need of several others on a cooperative nonprofit basis. Almost any hospital can easily maintain its own store of frozen plasma, which would be supplemented by a small amount of the dried material supplied by the drying center. Under favorable conditions, a large institution may assume the responsibility for supplying plasma in both frozen and dried states for neighboring institutions.

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A plan of this nature has been operating successfully for over eighteen months in the Cincinnati General Hospital under Dr. Hoxworth.[16] The service supplies the needs of fourteen hospitals with a total bed capacity of 4,235. A state-wide plasma service is being organized by Dr. James of the University of Vermont Medical College.[17]

The purpose of this paper is to describe a method and an apparatus for the drying of plasma from the frozen state by low temperature water vapor condensation in vacuo suited for the plan just mentioned.

While the essential details of construction are given, no attempt is made to describe the apparatus minutely. Likewise, no attempt is made to give details of performance under all conditions of temperature, vacuum, etc., nor to justify many statements made concerning newer methods employed, such as the shell freezing at relatively higher temperatures, etc.

These phases of the work will be covered in subsequent papers.

A DISCUSSION OF PREVIOUS APPARATUS AND BASIC PRINCIPLES INVOLVED

Up to the present, all of the various apparatus described for the desiccation of plasma from the frozen state have been either too small for practical use or too expensive if large enough to serve the purpose. Some apparatus, although inexpensive to purchase, have proved a financial burden because of the operating expense and the need of much technical supervision. Most of these apparatus have been recently critically reviewed by Harkins.[2]

Of all the methods in use, we feel that desiccation from the frozen state in the final container is preferred, because this method provides for maximal preservation of all elements of the plasma, maximum solubility, minimal opportunity for contamination during the process of drying, and regeneration with 0.1 per cent citric acid prior to use.

It is important to note that the pH of plasma dried from the frozen state and restored with distilled water varies from 8.2 to 9.3. The same material regenerated with 0.1 per cent citric acid will have a pH varying from 7.4 to 7.8.[15]

The use of low temperatures for the condensation of the water vapor removed from the frozen plasma is preferred because it is efficient and relatively inexpensive. In the apparatus to be described here, the same source of low temperature is used for both prefreezing of the plasma in shell form and for water vapor condensation during the process of drying. The source of low temperatures is obtained by expansion of compressed Freon-12 gas in evaporator coils or plates. It is based on the fundamental principles employed by the early workers in this field.[3] In addition, the successful operation of this apparatus involves five important facts, which previously had not been generally accepted or taken advantage of:

1. The temperature of freezing of plasma has little or no practical effect, within the experimental limits (minus 12 to minus 72° C.), on the quality of the final product.

2. The temperature at which water vapor condensation is carried out is not critical, within the experimental limits (minus 30° to minus 72° C.), provided that the rate of evaporation at any time during the operation is sufficiently

rapid to avoid thawing of the frozen plasma. This is readily accomplished by using a condenser possessing a condensing surface sufficiently large and by taking advantage of certain details of construction of the condensing chamber pointed out later.

3. The temperature of the condensing coil during the period of maximal sublimation may rise somewhat above the minimum mentioned (minus 30° C.) without adversely affecting the process, provided that during the last few hours of the process the temperature is maintained between minus 30° C. and minus 35° C. or below.

4. The temperature of the water jacket heating the plasma may be raised, during a portion of the process, to relatively high temperatures (80° C.) without impairment of any of the essential qualities of the resulting product, but with considerable shortening of the time of drying as compared with apparatus without proper thermic control.

5. The temperature of the plasma itself may be raised to relatively high temperatures (75° to 80° C.) without deterioration, provided that the amount of moisture present is relatively low. This occurs in the last few hours of the process.

The demonstration of these facts has allowed the development of an efficient, practical apparatus, which will fit the program of plasma drying mentioned above. The use of very low temperatures (about minus 70° C.), which has been emphasized for both prefreezing and drying, has been one of the main factors in the almost prohibitive cost of other apparatus. Several types of inexpensive standard refrigerating machines on the market are capable of producing constant temperatures in the nature of minus 40° C. and thus allow the construction of a satisfactory apparatus at a low cost.

In the construction of the apparatus, the following practical points were kept in mind:

1. The product obtained should, when regenerated with solvent, be as nearly as possible identical with the original material. The apparatus and process employed, therefore, must allow no degeneration to occur during the process of drying. The plasma obtained with the apparatus here described in all ways complies with the regulations set forth by the National Institute of Health.

2. The apparatus must be so designed and the operation so controlled as to be constant, thus insuring a uniform product. It must be remembered that drying from the frozen state may be achieved in a great many ways and with much simpler apparatus. However, mechanical controls of temperature, recording devices for temperature and pressure, and other refinements of construction play an important role in insuring a uniformly good product.

3. The apparatus must be simple and economical in its operation, as much as possible automatic, so as to need a minimum of supervision once under way. It should provide facilities for shell freezing as well as for drying. To achieve simplicity and economy of operation, the apparatus, paradoxically, has to be relatively expensive and complicated.

It has been pointed out that drying from the frozen state may be achieved with a simple and inexpensive apparatus.[4] Such an apparatus, however, has a

limited capacity, requires much technical supervision, and is high in operating cost. The apparatus to be described here was so designed as to have a capacity large enough for the need of several hospitals. It requires very little attention during the operation, and the operating expense is negligible.

Thus the standard apparatus here described freezes and dries 24 units of citrated plasma of 300 c.c. each daily (7.2 liters), or 8,760 yearly (2,628 liters). The total cost of electric energy for shell freezing and drying is well under $.02 per unit, or less than $175.00 yearly. For the same operation, if carbon dioxide ice is employed, the yearly cost of refrigeration would be about $3,006.00, or considerably more than the entire cost of purchase of the apparatus.*

In order to evaluate properly the construction and performance of this apparatus, it is necessary to review the general basic principles of drying biologic substances. The Bordas and d'Arsonval apparatus,[3b] as reconstructed by us, demonstrates these basic principles in a simple form (Fig. 1). It consists of two glass flasks connected by a T-tube. One flask (A) contains a thin layer of material to be dried. The other flask (B) is the condenser. The T-tube is connected to a vacuum pump by means of a rubber tube (C), which may be closed by a stout pinch clamp. The glass tube connecting the two flasks must be of sufficient size to allow free passage of water vapor from A to B. Flask A is immersed in water kept at plus 15° C., and Flask B, acting as condenser, is cooled by immersion in carbon dioxide ice-alcohol mixture, maintaining a temperature of about minus 70° C.

Containers A and B are stout-walled Erlenmeyer flasks of about 500 c.c. capacity. Under these conditions, when 30 c.c. of plasma are introduced in Flask A, "snap" freezing, from the heat loss due to rapid evaporation, occurs in about five minutes after starting of the vacuum pump; thereafter, drying proceeds by sublimation of water from the frozen state. The water vapor is condensed on the cold walls of Flask B as fast as it is formed; thus it is not allowed to mix with the oil of the pump, which would rapidly reduce the efficiency of the pump. The dried plasma thus obtained appears in the form of irregular scales, has a residual moisture of less than 1 per cent, and is readily soluble.

Practically all of the various types of apparatus in general use for drying under a vacuum are based upon variations in the method of condensing the water vapor.

In the original apparatus used by Bordas and d'Arsonval, liquid air or carbon dioxide snow-acetone was used as refrigerating agent.

Vansteenberghe[3a] used sulfuric acid to absorb the water vapor instead of low temperature; Reichel[5] and Flosdorf and Mudd[6] used an apparatus similar to that of Bordas and d'Arsonval and CO_2 ice as the refrigerating agent for the "lyophile" process; later Flosdorf and Mudd[7] returned to the use of a chemical dehydrating agent and employed calcium sulfate in their "cryochem process"; Hill and Pfeiffer[8] used silica gel in their "adtevac" process.

Elser[3f] was the first to report the use of coils refrigerated by the expansion of compressed Freon-12 gas for condensation of water vapor. In actual opera-

*The cost of CO_2 ice is computed at $.03 per pound, the lowest prevailing price when purchased in large quantities. The amount of dry ice required is based upon our experience with a similar apparatus.[12]

tion, minus 34° C. to minus 36° C. were the lowest temperatures attained. Elser pointed out that the loss in efficiency from the change of the condenser temperature from minus 70° C. to minus 32° C. was relatively slight, roughly 5 per cent.

The failure of the mechanical refrigeration system of Elser has generally been laid to the too small differential in temperature between the substance being dried and the condensing surface. This is not confirmed by our findings, since satisfactory drying with a residual moisture of less than 1 per cent has been obtained with a maximal differential temperature of only 65° C.

We have already pointed out that the temperature of prefreezing is not critical within the experimental limits (minus 12° C. to minus 72° C.). With the apparatus here described, prefreezing and drying are carried out at much higher, and therefore much more convenient and economical, temperatures than generally employed without essential loss of quality of the material being dried.

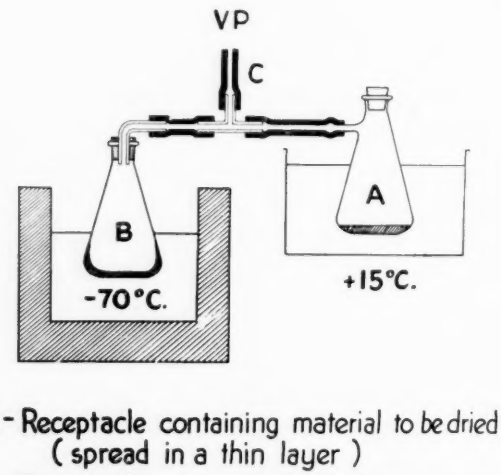

Fig. 1.—Bordas and d'Arsonval Apparatus for the drying of easily alterable substances.

Greaves and Adair[9] likewise found that the lowering of the condenser temperatures lowers the temperatures of the frozen material being dried to a very slight extent only. They successfully employed as refrigerating agent methyl chloride, and obtained temperatures of minus 52° C. to minus 42° C.

Greaves and Adair state that with temperatures of minus 45° C., the percentage of water remaining at the end of desiccation is much less than when higher temperatures are employed. We have found that with the water jacket of the drying chamber at plus 35° C. to plus 36° C. and temperatures of condensation of minus 30° C. to minus 35° C., the residual moisture of the resulting material is constantly less than 1 per cent.

The method employed for the determination of the residual moisture is that described by the National Institute of Health, i.e., drying of the material in high vacuum over phosphorus pentoxide to constant weight.

The great advantages in the employment of relatively high temperatures of water vapor condensation are, essentially, simplicity of operation and economy, since the heat neutralizing capacity of a compressor falls rapidly with the lowering of the temperature of condensation.

There are two methods of drying under vacuum which do not employ a condenser. One of these, the "desivac" machine,[10] allows the water vapor to enter the vacuum pump and constantly removes the water from the oil by means of a high speed centrifuge. The high cost of this apparatus and the relatively long drying time make its operation uneconomical. Another method produces a vacuum by means of multiple steam jets, in which case the water vapor escapes with the steam, thus eliminating the need for a condenser. This method is particularly suited to large quantity production on a commercial scale.[11]

With the possible exception of the method of Greaves and Adair, all of the above-mentioned methods require large and expensive apparatus to dry enough plasma to consider the process practical. In addition, the use of chemical desiccants requires redrying of the desiccant each time it is used, an operating entailing per se considerable time and expense.

An apparatus for the drying of plasma from the frozen state by low temperature water vapor condensation in vacuo, previously described by us,[12] was based on the principles laid down by Bordas and d'Arsonval and on the work of Shackell,[3d] Harris,[3c] and Rogers.[3e] In the construction of the drying chamber, advantage was taken for proper heat distribution to the material being dried of copper split cylinders or shells enveloping each bottle. These shells, properly fastened together, formed also a desirable method of handling the bottles during the loading of the drying chamber, making the operation easy and rapid. In the construction of the condenser, we took advantage of details of construction pointed out by Reichel[5] and by Flosdorf and Mudd.[6] This condenser was externally cooled by the use of alcohol-carbon dioxide ice mixture.

Routine use of this apparatus pointed out that while the drying chamber proved fairly satisfactory, the condenser was rather crude in design and expensive in operation, particularly because of the cost of carbon dioxide ice and the necessity for almost continuous supervision. The presence of a secondary condenser made the apparatus difficult to handle and proper insulation an almost impossible task with the result that much of the low temperature produced by the carbon dioxide ice was wasted. The time of drying was three days or more.

An attempt was made to eliminate the use of dry ice by cooling the condenser by the use of a low temperature, two-stage (cascade) compressor using Freon-12 (or propane) and ethane as refrigerants and of a circulating brine system giving a condensing temperature of about minus 60° C. This apparatus proved too bulky, expensive, and unreliable in performance.*

Our early choice of low temperatures, in the neighborhood of minus 60° C. to minus 70° C. for the operation of this condenser as well as for the prefreezing of the plasma, was suggested by the generally held idea that such low temperatures were essential for the proper preservation of the product as well as for the proper functioning of the apparatus.[5, 6]

*Better two-stage compressors are built today, but they do not offer any practical advantage by the use of lower temperatures to offset the greater cost of purchase and maintenance.

DESCRIPTION OF NEW APPARATUS

The construction of a condenser with refrigerated coils within the condenser chamber was next attempted. Coils within the condenser are more efficient because of the excellent insulation provided by the high vacuum. This condenser was and is being used with a drying chamber similar to the one previously described.

The standard apparatus described here is capable of drying, in less than twenty-four hours, twenty-four lots of plasma each containing 300 c.c. in a 400 c.c. container. Thus the capacity is 7,200 c.c. in twenty-four hours. The residual moisture of plasma dried in this apparatus is consistently well below 1 per cent of the weight of the remaining material, assuring adequate preservation and rapid solution when the plasma is restored with solvent.*

It is to be noted that the time of drying depends upon several factors. The most important are as follows:

1. The thickness of the shell of the frozen material. A thinner shell dries much more rapidly than a thick one.

2. The amount of heat applied to the frozen material in the process of drying. The more heat applied, the more rapid is the process. Details of the application of heat will be elaborated upon separately.

3. The diameter of the neck of the bottle. The larger the opening, the more rapid the process within certain limits.

4. The type of protection used to maintain sterility. In our method sterility is adequately maintained by covering the individual bottles with a large cuff made from two layers of 40-mesh gauze. This offers some resistance to the flow of water vapor and, therefore, lengthens the time required for drying. It is felt, however, that this is a desirable precaution against contamination.

The complete apparatus described here is used for prefreezing (shell freezing) the plasma and for drying the plasma from the frozen state. It consists of four main parts: (1) a mechanical shelling apparatus, (2) a drying chamber with a water jacket, (3) a condenser with a mechanically refrigerated coil, and (4) a vacuum pump.

1. *Shelling Apparatus* (Fig. 2).—This consists of an insulated metal pan, containing the cooling coils and the apparatus for rotating the bottles containing the plasma.

The cooling coil is formed of a series of straight copper tubes. The two center tubes are shorter to make room for a propeller which circulates the cold alcohol. A plate type evaporator of suitable capacity may be used instead of coils. The shelling pan is connected with a reservoir for the alcohol. Before use, alcohol is pumped into the shelling pan. At the end of the process, the alcohol is returned to the reservoir by gravity. The shelling pan contains a mechanical device, capable of turning twelve bottles at the rate of one-half to one rotation per minute. The arrangement of the rotating wheels is so that the bottles will be immersed in the cooled alcohol about 12 mm.

*The standard apparatus here described is constructed by the Precision Scientific Company of Chicago.

2. *Drying Chamber* (Fig. 3).—The drying chamber consists of a heavy brass cylinder (22.5 cm. in diameter and 70 cm. high) surrounded by a water jacket. It is closed on top by a heavy steel lid, which rests on a rubber gasket. The lid may be tightened by threaded lugs and wing nuts. The opening for the connection with the condenser is in the middle of the base of the drying chamber. This location constitutes an essential feature of the apparatus. It is flush with the base itself and is 5 cm. (2 inches) in diameter. The connection with the condenser is made by a short copper pipe, 5 cm. (2 inches) in diameter.

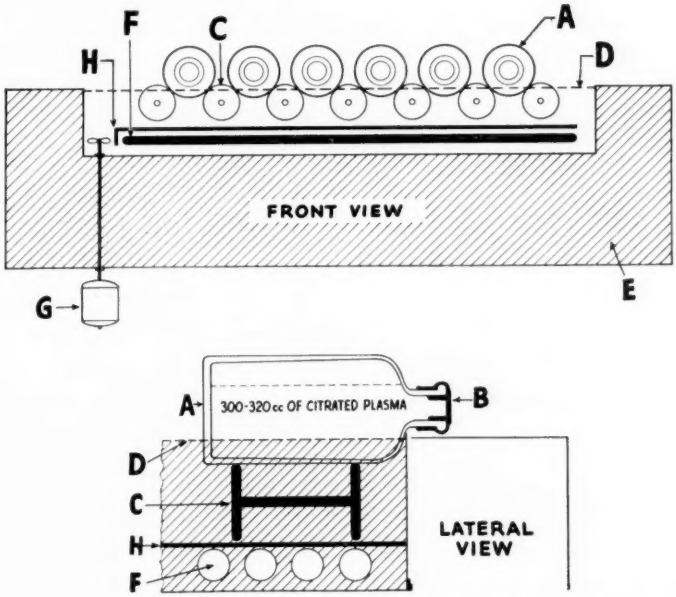

Fig. 2.—Apparatus for shell freezing with circulator for refrigerated alcohol (schematic). *A*, 400 c.c. bottle; *B*, rubber stopper; *C*, rubber-tired rollers; *D*, refrigerated alcohol level; *E*, insulated metal pan; *F*, evaporator coils; *G*, circulator; *H*, baffle board.

The drying chamber is surrounded by a water jacket formed by a cylinder of rustproof metal fixed to the flange of the inner chamber. The water jacket is 30 cm. (12 inches) in diameter and 90 cm. (36 inches) overall in length. It is provided with an electrical heater, fitted to the bottom and capable of maintaining the water at any desired temperature up to 80° C. This heater is controlled by a thermostat and relay. The temperature may be adjusted by setting the thermostat. The water bath is connected by pipes to sources of hot and cold water.

The dry chamber is fitted with four copper baskets. Each basket is composed of an outer split cylinder of 24 oz. copper approximately 22.5 cm. in diameter (9 inches) and 20 cm. in height (7⅞ inches). The lower border is turned in 12 mm. (½ inch). This strengthens the cylinder and holds the plasma bottles in place. This large cylinder contains a series of six smaller split copper cylinders welded to the inner surface. These are 11.8 cm. high (4⅝ inches) and are of the proper diameter to grip the plasma bottle tightly. The basket is completed by a transverse bar and springs, which maintain proper expansion

of the outer cylinder, and by a series of copper wedges, which assist in increasing heat condution as well as the contact of the copper shells with the bottles.

A recording thermometer is provided to determine the temperature of one of the copper shells of the lower basket. It has a range from minus 10° C. to plus 80° C. The bulb of the thermometer is fixed to the bottom of the drying chamber and is in direct contact with two of the copper cylinders. Its reading is affected by the temperature of the bottle of plasma in the copper cylinders. A dial thermometer shows the temperature of the water bath. When the temperature shown by these two thermometers remains within 42° C. for two hours or more, the plasma may be considered dry.

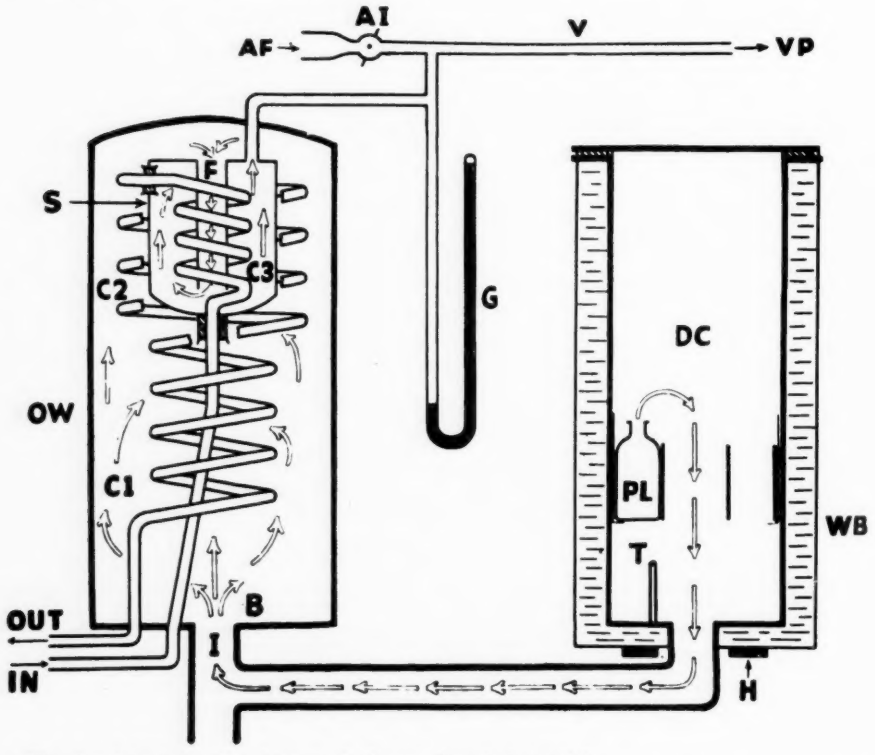

Fig. 3.—Apparatus for drying of plasma (semischematic).

IN, Inlet of evaporator; *OUT*, outlet of evaporator; *OW*, wall of condenser chamber; *C1-C2-C3*, portions of the evaporator (refrigeration coil); *I*, inlet for water vapor; *F*, funnel-like opening conveying water vapors from the general condenser chamber to the coldest portion of the evaporator coil, *C3*; *B*, base of the condenser chamber; *G*, vacuum gauge; *AF*, air filter; *AI*, air inlet valve; *V*, vacuum line; *VP*, vacuum pump; *H*, 750 Watt heater; *WB*, water bath; *DC*, drying chamber; *Pl*, plasma; *S*, shield of condenser; *T*, bulb of recording thermometer.

One of the difficulties in the drying of plasma from the frozen state is the proper application of heat to the frozen plasma to counteract the cooling effect of rapid evaporation. If no heat is applied, the process will require several days, while properly controlled heating reduces the time to less than twenty-four hours. Heat from the water bath around the drying chamber is conducted to the plasma very satisfactorily by means of the copper baskets just described.

3. *Condenser* (Fig. 3).—In the construction of the condenser there are two essential features:

1. The large water vapor inlet is located in the lowest part of the condensing chamber.

2. The refrigerated coils are so arranged that by means of properly placed cylindrical shields, the water vapor is made to travel a long distance and come in contact with a large condensing surface. The last portion of the coil with which the vapor comes in contact is the coldest. This assures condensation of all of the water vapor and prevents any water from mixing with the oil of the vacuum pump.

The water vapor enters the condenser from the bottom. This is very important, because it allows slower diffusion of the water vapor and allows maximal condensation on the first set of coils with which the vapor comes in contact. Under ordinary conditions, the greatest portion of the vapor is condensed on Coil C1. Some vapor escapes Coil C1 and reaches Coil C2. Here the vapor travels through a rather narrow space between the wall of the condenser and the cylindrical copper shield S placed inside of Coil C2. If a trace of water vapor escapes Coil C2, it must travel through a narrow funnellike tube (F) to reach Coil C3 which is enclosed in a copper box. The vacuum pump is connected to the condenser by means of an opening in the top of this box. The great bulk of the water vapor is condensed on Coil C1, a smaller amount on Coil C2, and only a trace on Coil C3 (Fig. 4). No water escapes to the vacuum pump. This has been determined by placing a copper U-tube 5 cm. in diameter in the vacuum line V during experimental runs. The U-tube was immersed in a bath of carbon dioxide ice and alcohol maintaining a temperature of minus 70° C. to minus 72° C. At the end of the runs no ice was found in the U-tube.

A Servel F-12, four-cylinder, ¾ h.p. compressor with dehydrator and oil separator is very satisfactory for cooling the coils in the shelling pan and in the condenser. The shelling pan and the condenser may be cooled simultaneously or individually. The F-12 gas should be pumped out of the coils before heat is applied in the condenser to melt the ice at the end of each run. The coils in the condenser will be maintained from minus 35° C. to minus 40° C. without a load, and from minus 30° C. to minus 35° C. while the drying is being accomplished. Occasionally during the period of maximal condensation, the temperature may be a little higher.

The condenser chamber itself is made of copper sufficiently strong to withstand a high vacuum. The total length of the refrigerated coil is 13 m. (44 feet), and the inner diameter 1.56 mm. (⅝ inch). A dial thermometer shows the temperature of the "In" coil of the condenser. This greatly assists in checking proper operation of the refrigerating compressor. An electric heater is provided in the condenser to melt the ice from the coils rapidly at the end of each drying run. The melted ice is then drained from the condenser through the opening in the bottom.

The copper box (S of Fig. 3) is so constructed that the ice formed on Coil C3 will drain out once it is melted. This is accomplished by means of a self-sealing valve surrounding the "In" coil as it enters the box. The valve consists of a short length of copper tube insulated from the rest of the box and approx-

imately 2 mm. larger in diameter than the refrigerated coil passing through it. When the drying process is initiated, water vapor very soon freezes around the refrigerated coil passing through the valve, and so seals it. When the ice is melted, the valve is open and allows water from Coil C3 to drain from the condenser.

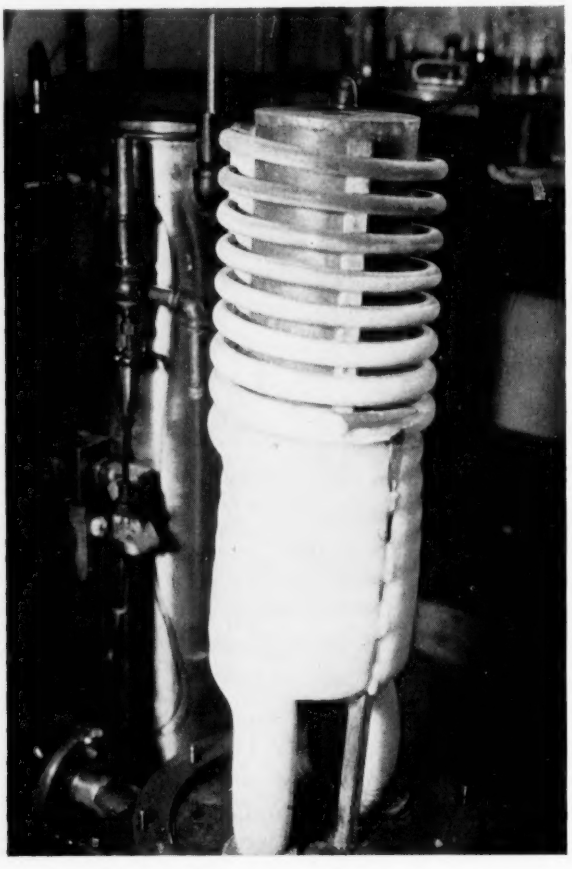

Fig. 4.—Ice formation on the condensing coil after a standard run (about 7200 c.c.)

4. *Vacuum Pump and Gauge.*—The large Welch Duo-Seal or the Cenco-Megavac with ½ h.p. motor has proved adequate. The pump must have a capacity sufficient to evacuate the system to a pressure of less than 2500 microns in ten minutes and 500 microns in less than fifteen minutes with the temperature of the condensing coils at minus 30° C. to minus 40° C.

A Pirani type vacuum gauge, such as the Truvac Gauge, Model 12, is satisfactory. It should be graduated from 2500 microns to 0 microns. A MacLeod type mercury gauge is also satisfactory. It is desirable to have, in addition, a rough dial type vacuum gauge, reading from atmospheric pressure to zero, to be able at a glance to watch the initial rate of evacuation of the system when a drying run is being started. This ascertains, in the first minute or so, that all outlets have been properly closed.

The entire apparatus is compactly assembled in a single cabinet and all switches, valves and recording dials and instruments are placed on a single panel (Fig. 5).

Fig. 5.—Standard shell freezing and drying apparatus. In the rear can be seen the shelling pan; in the right anterior corner the drying chamber, and in the middle front the panel board.

MECHANICAL SHELL FREEZING AT MINUS 20° C. TO MINUS 35° C.

We had previously found that the choice of low temperatures in the neighborhood of minus 60° C. to minus 70° C. for rapid prefreezing of plasma does not offer any practical advantage. A mechanical apparatus which gave good results[1a] was developed for shell freezing at minus 40° C. It was found, however, that shell freezing at temperatures higher than minus 40° C. was not possible with continuous rotation, because plasma would become supercooled at minus 6° C. to minus 12° C., and would then "snap" freeze, with slushing and formation of an irregular shell, with large lumps which greatly interfered with drying later on.

In attempting to shell freeze plasma at temperatures between minus 20° C. and minus 35° C., it was noted that if early initial local freezing occurred in contact with the inner surface of the bottle, shell freezing continued thereafter without lumping. It appeared necessary in the course of experimental work to produce a relatively rapid cooling of plasma to about plus 10° C. from room temperature followed by local freezing, to avoid supercooling of the entire mass and slushing following "snap" freezing. This is accomplished with the fol-

lowing technique: The plasma bottle is rotated slowly (½ to 1 r.p.m.) with 12 mm. immersion in alcohol cooled to minus 30° C. to minus 35° C. for a period sufficiently long to bring the temperature down to about plus 10° C. The length of time required for this operation depends chiefly on: (a) the temperature of plasma, and (b) the temperature of the brine. It varies from three to ten minutes. With plasma at plus 25° C. and the brine at minus 30° C., about ten minutes are sufficient. After the period of cooling, the rotation is stopped long enough to cause the lower portion of the plasma to "snap" freeze to a depth of about 3 to 4 mm. This requires two to four minutes. The phenomenon is, as a rule, clearly visible. It is important to remember that during this period of time, the bottle of plasma must not be disturbed, and that the depth of immersion of the bottle in the cooled alcohol must be 10 to 12 mm. only. Occasionally, and for no apparent reason, one bottle may take considerably longer to show initial "snap" freezing. It must not be disturbed until "snap" freezing has occurred. It is desirable to obtain a temperature of minus 35° C. or a little lower before placing the bottles in the shelling pan.

When the local "snap" freezing has occurred in all of the bottles, the rotating motion is resumed, and shell freezing occurs at a regular rate and very evenly. With a temperature varying between minus 35° C. and minus 25° C., it requires one hour and fifteen minutes to one hour and twenty-five minutes for complete shell freezing of 300 c.c. of plasma. The quality of the material thus frozen is practically identical with that obtained from shell freezing at minus 70° C. The material thus obtained is readily recognized as it shows a longitudinal portion of microcrystalline, homogeneous aspect, lighter in color, and about 4 to 5 cm. wide and 3 to 4 mm. thick. The remaining portion shows a macrocrystalline structure. This aspect is clearly retained after drying from the frozen state (Fig. 6).

When the plasma is shelled, the bottles are placed in a storage cabinet at minus 20° C. to minus 30° C. to cool. It takes about four hours for the frozen material to cool from about 0° C. to minus 20° C. The plasma thus shelled can remain in cold storage for an indefinite period of time.[13]

About one hour before initiating the drying process, the copper baskets are placed in the low temperature cabinet to cool. At the same time, the bottles of shell-frozen plasma are removed, one by one, from the storage cabinet and, under aseptic precautions, the rubber stoppers are replaced by large sterile gauze cuffs formed of two layers of 40-mesh gauze. The bottles are now placed in the copper baskets, and allowed to cool for about one-half hour at minus 20° C. to minus 30° C.

TESTING AND OPERATION OF THE APPARATUS

Before each run the apparatus should be tested to be sure it will produce a satisfactory vacuum and low temperature. The drying chamber and the inlet valve are closed, and the compressor is started and allowed to run until the thermometer on the condensing coil registers minus 30° C. or less. The vacuum pump may then be started and a vacuum of 500 microns (Truvac gauge) or less must be obtained in fifteen minutes or less. If this is accomplished, the vacuum pump is shut down, the vacuum is released, and the drying chamber opened.

The copper baskets containing the shell-frozen plasma are rapidly placed in the chamber, the lid is closed, and the vacuum pump started. This operation can readily be accomplished in about two minutes. A pressure of 500 microns or less should be attained in fifteen minutes or less to avoid danger of thawing. The water in the water jacket of the drying chamber must be cool, not over 25° C.

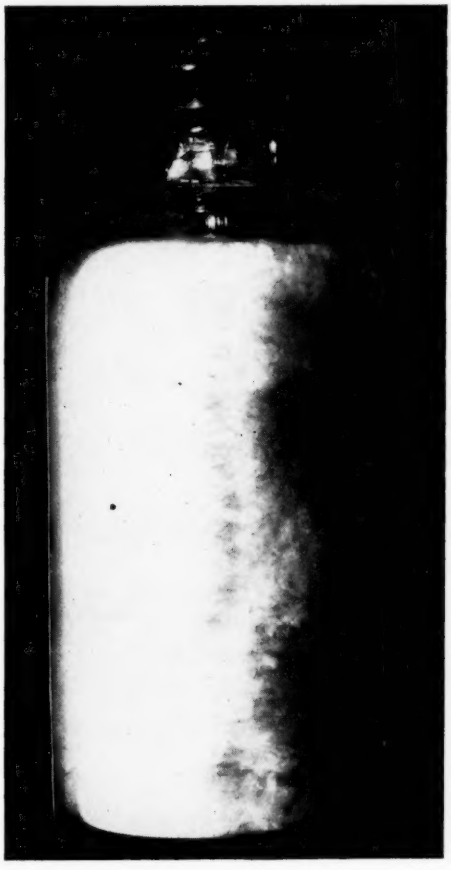

Fig. 6.—Bottle of dried plasma showing on the left homogeneous aspect corresponding to the portion of plasma "snap" frozen, and on the right the gross crystalline structure prevailing in the remaining portion.

The operation is allowed to proceed for one hour. The heater of the water jacket is then turned on with the thermostat set at plus 50° C. Drying is allowed to proceed until the thermometer recording the temperature of the plasma has been for two hours or more within two degrees of the temperature of the water in the water jacket. The vacuum pump is then stopped, and filtered air is allowed to enter slowly through the inlet valve. This air passes through the condenser before it enters the drying chamber and thus is dried. The opening of the inlet valve is covered with 40 to 50 layers of gauze to effect a good filtration of air, sufficient per se to minimize danger of contamination. The air should be allowed to enter slowly, taking about five minutes for the entire operation. The copper baskets are removed one at a time, and the bottles promptly stoppered.[12] The bottles are then evacuated, and the rubber stoppers are covered

with gel caps. This method of closure is sufficient for preservation up to three years. If the preservation is to be for longer periods of time, the material is vacuum-sealed in tin cans.[14]

The time of drying with a continuous temperature of 50° C. in the water jacket is less than eighteen hours. When it is desirable to reduce the drying time, the temperature of the water jacket may be increased up to 80° C. for a period of time.

The dried plasma obtained with the apparatus and technique outlined above is a light, porous material, with a distinct crystalline structure, and a volume slightly less than that occupied by the frozen plasma. It may be readily reduced to a fluffy powder by shaking, in which case it occupies about one-third of the original volume. The powder thus obtained is readily transferred to another receptacle for purpose of residual moisture determination or of combining the content of two or more bottles. The color of the dried plasma is light amber, when the original liquid plasma had a hemoglobin content of less than 5 mg. It is very soluble in water. When solvent is introduced by vacuum suction, dried plasma containing 17.5 Gm. of plasma proteins (the equivalent of about 300 c.c. of fresh citrated plasma) dissolves entirely in 250 c.c. of distilled water or 0.1 per cent citric acid solution in less than one minute, including the time consumed by the introduction of the solvent.

The resulting fluid is stable at room temperature for at least several days; it has a maximal content of complement, and a maximal content of prothrombin if regenerated with 0.1 per cent citric acid solution.[15] The use intravenously of properly prepared material causes no untoward reactions, save an occasional (less than 1 per cent) urticarial reaction.

The size of the apparatus here described is considered best for the purpose intended, namely, for large hospitals to dry plasma for their own use and for neighboring institutions. Both smaller (1800 c.c. daily capacity) and larger (21,600 c.c. daily capacity) units have been successfully operated, however. When larger units for commercial operation are built, it is desirable to institute automatic temperature control, for minimum drying time with the least technical supervision, by the installation of a cam-type combination thermoregulator and recording apparatus, such as the one furnished by the Foxboro Company, Mass.

In addition to human plasma, serum, guinea pig complement, bacterial cultures, milk, many fruit juices, culture media, and even meats and other products have been dried with the machine here described with excellent preservation of all essential properties including flavor and vitamin content.

The advantages of the apparatus here described over other apparatus are:

1. Both prefreezing in shell form and drying from the frozen state are accomplished by employing the same economical source of low temperature.

2. The apparatus is very simple in operation and almost entirely automatic.

3. It yields constantly a product in every way satisfying the requirements set forth by the National Institute of Health.

4. The time of drying is considerably shorter than that obtained with other methods.

5. The total cost of operation per unit is very small.

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A NEW FIBRINOLYTIC METHOD FOR THE ASSAY OF PROTEASES AND ITS APPLICATION TO AN ANALYSIS OF COAGULATION PHENOMENA IN RABBIT BLOOD*

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SINCE 1939, this laboratory has been contributing evidence for a new theory[1] of blood-clotting, based upon the dominant role of serum-tryptase, a proteolytic enzyme, long recognized by biochemists (Oppenheimer[2]). Even in the complete absence of formed elements, this plasma enzyme normally performs actions which we are able to duplicate with *crystalline* (pancreatic) trypsin, in vitro. These actions are (1) thromboplastic conversion of prothrombin to thrombin[16, 3] (whence the designation "thromboplastic enzyme"[4]), (2) digestion of prothrombin[5] and thrombin[6] (whence the term "progressive" antithrombin to distinguish the latter from "immediate," neutralizing and reversible[7] inhibition of thrombin, for example by heparin and its albumin-fraction "co-factor"), (3) lysis of fibrinogen and fibrin[8] (whence the term "fibrinolysin" is superfluous). Clot retraction is probably just the initial phase of tryptic digestion of the clot, and natural fibrinolysis plays an important role in the resolution of (blood) clots in vivo.[3] Ferguson's claim that a plasma deficiency of available tryptase is the true explanation of the coagulation delay in *hemophilia*,[9] has been confirmed by Feissly,[10] with the aid of a gelatine liquefaction technique. Continued search for a method whereby this and other problems relating to the tryptase factor could be put to a quantitative test, has yielded a new assay technique, as follows:

ASSAY OF TRYPSIN BY FIBRINOLYSIS

(*Relative Turbidimetry With the Photoelectric Colorimeter*). *Method.*[11]—Varying amounts of trypsin are added to alkaline (buffered) mixtures of fibrinogen and thrombin and lysis of the clots formed is timed by the relative positions of the curves charted with the aid of the Evelyn apparatus.

Reagents.—The test requires a stable fibrinogen and a stable thrombin. Citrated blood plasma contains a natural lytic factor (serum tryptase), but this apparently deteriorates on keeping. Hence, the *fibrinogen* (F) is routinely prepared by three precipitations with $(NH_4)_2SO_4$ (¼ sat.), from refiltered (dog) plasma, several days old. The preparation is suitable if it shows no visible lysis (in the control) during the course of the experiment. An excellent stable *thrombin* (T_6) is obtained by a 1:100 dilution of Parfentjev's[12] "rabbit clotting globulin" (Lederle Laboratories). In an optimal series of *trypsin* dilutions, the highest enzyme concentration used causes fibrinolysis in one and one-half to two hours and the weakest in six to twelve hours (a matter of convenience). The strongest typsin should not significantly interfere with clot formation. All reagents contain 0.9 per cent NaCl (plus thymol) and

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the fibrinogen is buffered with barbiturate,[6] borate, or other suitable buffer. A routine pH of 7.5-7.75 is sufficiently alkaline for the enzyme action, without increasing the translucency (impairing turbidity) to the extent seen with unduly alkaline mixtures. The following amounts of reagents are mixed in the order cited: .5 c.c. *buffered* fibrinogen + 1 c.c. saline (= control for experiments on inhibitor or other agents it is desired to study) + 3 c.c. thrombin + 1 c.c. trypsin. In order to minimize lysis of the fibrinogen and thrombin, the enzyme is added five to ten seconds before the onset of clotting.

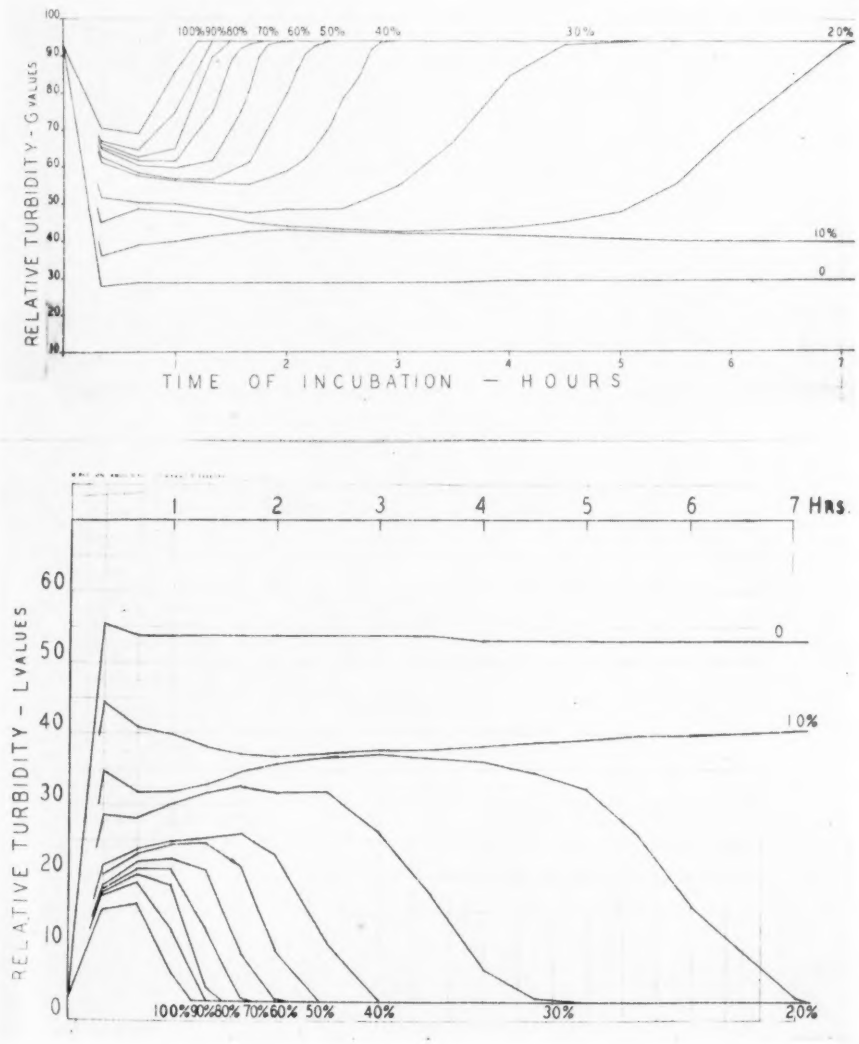

Fig. 1.—Assay of crystalline trypsin (standard X.T.) by fibrinolysis. Relative turbidimetry with the Evelyn photoelectric colorimeter. 1/10 vol. of 1:4000 X.T. = 100%. Temp. = 22° C., pH = 7.5.

Data.—The data presented graphically in Fig. 1 form a typical reference series on a crystalline trypsin preparation, 1 mg. of which analyzed 4.24×10^{-3} $[T.U.]_{Mb}$ by the Anson and Mirsky method.[13] Ten per cent fractions of a stock (100 per cent = 1:4000) were used in one-tenth vol. (v. supra), hence the *final* concentrations vary from 1:40,000 down to 1:400,000. The curve for 1:400,000

(10 per cent) is incomplete since fibrinolysis in this case required 15 to 16 hours, as compared with 7 hours, 5 hours, 3 hours, 145 minutes, 130 minutes, 110 minutes, 90 minutes, 80 minutes, 72 minutes, for the 20 per cent to 100 per cent, respectively. The control was completely unaffected in 24 to 48 hours. Temp. = 22° C., pH = 7.5 (checked in all tubes, with glass electrode, at completion of fibrinolysis). Clotting-time (C.T.) = 25 seconds: the same in the control and all the enzyme-containing mixtures.

Instrument data.—Green filter (540). Galvanometer (G) values: (1) Blank (10 c.c. saline) = 1000; (2) initial reading (all tubes) = 930; (3) final reading (all tubes, except control) = 940.

The slight persistent turbidity is due to a very faint opalescence, probably associated with traces of lipids or thrombin impurities which are still present after fibrinolysis.

Comment.—The absence of C.T. change is evidence that the thrombin and fibrinogen are not significantly attacked by the trypsin concentrations used. However, the relative turbidity is progressively lessened as the enzyme strength is increased (see Fig. 1) and this is probably a more sensitive indication of a minor degree of fibrinogen destruction. Such fibrinogenolysis as may occur enhances the sensitivity of the turbidimetric test, but lessens the significance of the actual values. It is not the shape of the curves which is significant, but their relative positions along the time axis. A simple charting of galvanometer scale readings (G) serves the purpose of *timing* the fibrinolysis just as satisfactorily as the corresponding "photometric densities" (L), which require computation by the formula $L = 2 - \log G$. Both methods are illustrated in Fig. 1. The steep terminations of the curves for the various mixtures are clearly separated by marked time differences. Hence, the strength of any "unknown" solution of similarly-acting lytic agent (suitably diluted) can be assayed from the position of its end-curve with reference to those for the standard trypsins. A good commercial trypsin of known potency is just as serviceable as the crystalline enzyme.* The 10 per cent "limit of error" is extremely liberal, particularly with the longer-acting (i.e. weaker) trypsins.

In preliminary testing for suitable dilution range, simple visual timing of fibrinolysis is adequate and the photometer can also be dispensed with if the "lytic times" are sufficiently long and well separated. Turbidimetry is invaluable, however, for enhancing the reliability of observation and permitting assays to be made with the greatest possible accuracy in the shorter time intervals. The absolute practical sensitivity of the fibrinolytic assay is of the order of 1:1,000,000 final crystalline trypsin ($=4.24 \times 10^{-6}$ $[T.U.]_{Hb}$), which makes the method especially applicable to quantities of enzyme too small to be assayed by all but the best (Anson and Mirsky[13]) proteolytic methods. It offers three improvements over the method used for bacterial "fibrinolysin" by Christensen:[14] (1) standard reference enzyme, (2) greater accuracy of the photoelectric timing, (3) an absolutely stable (tryptase-free) fibrinogen is not essential. A trace (0.1 per cent) of citrate is not objectionable, but tryptase inhibitors must be absent.

*Further detail as to standard "units" and reagents is given in a note to Federation Proc. (1943). A Newer Method, by Lysis of Fibrinogen, Proc. Soc. Exper. Biol. & Med. 52: 243, 1943.

Effects of Varying Fibrinogen Concentration. Method.—Five strengths of a fibrinogen solution (v. supra), which yielded 200 mg. of *fibrin* per 100 c.c. (dry weight, by a modified Gram method), were clotted by T_6 (as above) in the presence of 1/10 volume of 1:4000, 1:8000, 1:16000, 1:32000 X.T., along with a control (without trypsin) for each series. Table I summarizes both the clotting-times and the "lytic times," determined with the aid of the photoelectric colorimeter.

Comment.—As expected, the rate of fibrinolysis is speeded up by lowering the substrate (fibrin) concentration. The clotting-times indicate that the 0.2 per cent fibrinogen was a little too strong for optimal clotting and gave slowest lysis with both strongest and weakest enzyme concentration. The 0.0125 per cent fibrinogen was too weak, giving longer clotting-time (hence favoring fibrinogenolysis by the added enzyme) and insufficient turbidity. Nevertheless, the control remained stable for three days and the turbidimetric timing of the lysis seemed satisfactory. It was particularly noted that the weaker fibrins gave quicker lysis with both the strongest and weakest enzyme concentrations. The facts suggest a trace of *natural inhibitor* in the fibrinogen. With intermediate enzyme concentration (final conc. = 1:80,000, 1:160,000) the varying fibrinogen produced only minor differences in the lytic times. It may be concluded that a fibrinogen concentration of 0.05 to 0.1 per cent is optimal for the trypsin assay. With trypsin concentrations below 1:300,000 (final), somewhat weaker fibrinogens may be advocated.

Effects of Temperature and pH.—Our experience confirms the information given by Christensen, for bacterial "fibrinolysins," and the data need not be presented in detail. We find it convenient to carry out the lytic tests at room temperature (20° to 25° C.) and a thermostat water-bath is indicated only for timing lysis occurring more rapidly than two hours. Tryspin has a broad zone of optimal pH (8 to 9, Northrop) and considerable activity is retained well into the acid side of neutrality. The fibrinolytic method is adapted to the assay of any proteolytic enzymes which are active between pH = 6.5 and 8.5. Too great alkalinity lessens the "photometric density" of the fibrin gel, leading us to prefer a pH = 7.5 – 7.75 as the practical optimum for the method, albeit this is about the limit of usefulness of the barbiturate buffer recommended (since barbituric acid tends to precipitate from the buffer mixture).

TABLE I

EFFECTS OF VARYING FIBRINOGEN CONCENTRATION ON ASSAY OF TRYPSIN BY FIBRINOLYSIS. "LYTIC TIMES" AT 25° C., pH = 7.5

FIBRINOGEN CONC. (%)	0.2	0.1	0.05	0.025	0.0125
Trypsin conc. (final)					
1:40,000	75 min.	60 min.	45 min.	40 min.	35 min.
1:80,000	2 hr.	2½ hr.	1¾ hr.	1½ hr.	1¼ hr.
1:160,000	3½ hr.	4 hr.	3 hr.	3 hr.	2½ hr.
1:320,000	9 hr.	7½ hr.	5 hr.	5 hr.	3½ hr.
0*	∞	∞	∞	∞	∞
Clotting-times	30 sec.	25 sec.	25 sec.	26 sec.	55 sec.

*None of the controls showed any lysis in 3 days. The clotting-times are for the controls, but were not significantly modified by the addition of enzyme in the manner specified.

Use With Other Proteases.—The new method obviously suggests application to the study of all "tryptases" (Oppenheimer[2]) including those of plasma, leucocyte (and platelet), and tissue origin; also bacterial proteases and the

proteases in snake venoms (which may possibly have a bacterial origin), and papain. It is an easy way to control steps in the purification of such enzymes and their behavior with respect to small changes in pH, salt concentration, action of inhibitors, etc.

SERUM-TRYPTASE AND BLOOD-CLOTTING IN THE RABBIT

The following materials were tested: A, 10 c.c. citrated (0.38 per cent) rabbit plasma, diluted to routine volume (25 c.c.) with thymol-saline; B, same (1:2 dilution) citrated plasma clotted by *excess* of $CaCl_2$, the fibrin being removed by whipping with a glass rod, leaving the clear serum for the chloroform treatment (infra); C, (1:2) citrated plasma, clotted by 2 c.c. enzyme-free thrombin (1:100 dilution of Parfentjev's rabbit globulin), the fibrin likewise being removed; D, the fibrin from C, washed thoroughly in three changes of saline and resuspended in routine volume (25 c.c.) of saline; E, ten-hour-old serum from clotted whole blood. In order to remove trypsin-inhibitors (which will not be considered at this time), we employed the old method of Nolf, et al, (Tagnon[15]), shaking with one-tenth volume of chloroform. The (50 c.c.) bottles were centrifuged after three-quarters of an hour, the supernatant being left in contact with the $CHCl_3$ and samples removed 1, 6, 24 hours from start of treatment. Clotting-tests and controlled fibrinolytic studies (routine trypsin-assay method) are summarized in Table II.

TABLE II

CLOTTING AND FIBRINOLYTIC TESTS ON RABBIT PLASMA MATERIALS

	CHLOROFORM TREATMENT			CLOT RETN.	LYSIS OF CLOT	LYTIC TIME (TRYPTASE TEST)
	(A) 1 HR.	(B) 6 HR.	(C) 24 HR.			
A Plasma (citrated)	0 (24 hr.)	27 min. (+)	36 min. (+)	none	none	>4 days
B Serum (excess Ca)	135 sec. (++)	40 sec. (++)	20 sec. (++)	3½ hr.	3 + da.	40 hr.
C Serum (Thrombic)	1 hr. (+)	0 (24 hr.)	2 hr. (+)	2nd day	3 + da.	29 hr.
D Fibrin (from C.)	51 min. (+)	42 min. (+)	58 min. (+)	2nd day	3 + da.	20 hr.
E Ten-hour serum (whole bld.)*	24 min. (++)	–	–	none	none	>4 days
F Control (F + T_6)	35 sec. (++)	–	–	40 hr.	48 hr.	48 hr.

*0.1 c.c. serum + 1.9 F. A similar test with fresh (10 hr.) untreated serum clotted in 28 min.

TRYPTASE TESTS

Room temperature = 23° C. ± 2° C. pH = 7.75 (barbiturate buffer). 1 c.c. test-material in 10 c.c. fibrinogen-citrate (0.03 per cent)-buffer mixture. Standard trypsins: X.T. (v. supra) — 1:500,000; 1:1,000,000; 1:2,000,000 (final concentration) gave "lytic times" of 12½, 18, 24 hours, respectively, while the control (F) was stable for two days. Tests were made both with

the one-hour and the 24-hour samples, the latter with a weaker (1:3) fibrinogen. Except for a very slight increase in enzyme activity in *B* in the 24-hour, as compared with the one-hour sample, the data (including standards) were so similar that only the tests with the one-hour materials will be cited (Table II).

Data.—*A* and *E* showed no digestive activity in four days. Indeed, they were more stable than the control (F.) of 48 hours "lytic time," suggesting the presence of an *anti*-tryptase factor. In *D*, fibrinolysis was complete in 20 hours, with *C* not far behind (29 hours). *B* (one-hour sample) was weakly postive, with digestion about half completed in 36 hours, and quite clear in 40 hours (when the control was just beginning to lyse). All tests indicate very little enzyme and fall below the satisfactory working range of the method. Even so, the data are valuable and, allowing for the plasma dilution (final = 1:25), it can be stated with some assurance, that the rabbit plasma (serum) materials tested have a trypsin (X.T.) equivalence (by the fibrinolytic test) of less than 1:40,000. This is equivalent to .025 mg. X.T., or 1.06×10^{-4} $[T.U.]_{m}$ per c.c. Comparison with dog, human, and other plasmas will be deferred for a subsequent communication, but it may be stated that the *rabbit* data are significantly lower than in carnivorous species. This offers a rational explanation of the exceptional stability and enzyme-free character of Parfentjev's "rabbit clotting globulin," which makes it so excellent a thrombin for the assay test and other purposes.

Owing to the relative paucity of serum-tryptase, rabbit blood behaves somewhat differently from dog blood, in clotting experiments, under conditions similar to those employed by Tagnon.[15]

COAGULATION TESTS

Room temperature = 23° ± 2° C. Clotting-times for 1.0 c.c. weak fibrinogen solution *plus* 0.5 c.c. chloroform-treated materials. Character of clot (in parenthesis, Table II): ++ = complete (tube invertible); + = incomplete. The significance of the experimental data may be interpreted as follows:

1. When plasma is shaken with $CHCl_3$ and the tryspin-inhibitors thereby "neutralized," small amounts of serum-tryptase are activated. By its *thromboplastic action*,[16, 4] notwithstanding depression of the Ca-ions due to the citrate present, the enzyme activates a part of the prothrombin to thrombin. This increases to a low "peak" level and then decreases. We do not wish, at this time, to discuss the several possibilities which might account for the later decrease, but adsorption on fibrin, "immediate" anti-thrombins (i.e. "meta-thrombin" formation) and progressive thrombinolysis may be mentioned. The persistence of such a weak thrombin at all shows that tryptic thrombinolysis is negligible. Considerable unaltered prothrombin persists in the $CHCl_3$-treated plasma, as shown by (5-minute) recalcification of a sample and adding to fibrinogen, when solid clotting occurs in eight minutes. The recalcification produces no clot in the $CHCl_3$-plasma itself; neither does added thrombin, thus showing that the fibrinogen has all disappeared. It is, in fact, deposited as a fibrin coagulum (flocculant) adjacent to the chloroform layer in bottle A. No such appearance is seen in B — E. The thrombin formed by the tryptase action is sufficient to account for the fibrin deposit. The clots in the tests with

fibrinogen, although incomplete, were perfectly stable for three days. The important conclusion is that serum tryptase, in amounts too small to be detected by any of its other actions (viz., lysis of fibrin or thrombin, or their precursors) is, nevertheless, able to exert the typical thromboplastic action in the conversion of prothrombin to thrombin.

2. In recalcified plasma, thrombin production proceeds in the *natural* manner but is doubtless aided by the thromboplastic action of small quantities of tryptase liberated by the $CHCl_3$-treatment. This might not be true if the conversion of prothrombin to thrombin were completed prior to the addition of chloroform. In the present experiment, however, the conversion was definitely retarded and for an unusual reason, namely, too great an excess of Ca, occasioned by the inadvertent use of stock (N/2) instead of the customary (N/10) $CaCl_2$. Thus the preliminary plasma clotting required twenty minutes, instead of three minutes noted on repetition with N/10 $CaCl_2$. Thinking that the experiment might be instructive, the first sample was chosen for the $CHCl_3$ treatment and it did yield the pertinent observation that additional thrombin continued to form during the twenty-four hours of contact of the serum with chloroform. No evidence of tryptic thrombinolysis was obtained. The fibrinolytic test proves the paucity of tryptase, but the assay is definitely positive and in the clotting-test there was enough enzyme to produce clot retraction in three and one-half hours and the clot had almost lysed by the end of the third day, when observation was discontinued.

3. The addition of enzyme-free thrombin to citrated plasma produces complete conversion of fibrinogen to fibrin and unequivocal liberation of tryptase (see lytic tests). There is no evidence of any *immediate* thrombin formation from the plasma prothrombin. The slight excess of added thrombin is sufficient to account for the weak (60-minute) clot after the one-hour $CHCl_3$ treatment. By the end of six hours no thrombic activity is left, probably owing to destruction by the tryptase formed. After twenty-four hours a (weak) thrombic action reappears. This is attributed to the slow thromboplastic action of the enzyme on the prothrombin of the serum. That an abundance of prothrombin persists unaltered in the serum is clearly shown by a test in which a (30-minute) recalcified sample of the twenty-four-hour serum clots a test fibrinogen solidly in eighty *seconds*. The lytic tests reveal the small quantity of tryptase responsible for these effects. Retraction of the weak original clots, (*A*) and (*C*), occurred on the second day but lysis was far from complete by the end of the third day.

4. That well-washed fibrin contains traces of thrombin, which can be eluted with saline, is the basis for the old Gamgee-Howell method of thrombin preparation. The slight increase in the thrombin obtained after six hours is probably due solely to the longer period of extraction. Enzymic thrombinolysis may account for the falling off in thrombic activity in the twenty-four-hour sample. The persistence of such a weak thrombin in the presence of the serum tryptase revealed by the fibrinolytic tests is remarkable, however, and suggests that adsorption onto the fibrin protects the thrombin (to some extent) from the digestive action of small quantities of enzyme, which are obviously also adsorbed onto the fibrin clot and eluted with saline. The positive tests of *C* and

D show that the tryptase liberated by the $CHCl_3$ treatment is divided between the serum and the clot. Tagnon[15] was, therefore, well advised in shaking the *whole* clotting mixture with $CHCl_3$ in his fibrinolytic experiments and we propose to continue this practice in the clinical examination for human blood tryptase.

5. The serum experiments show a trace of persistent thrombin, which is to be expected in the absence of demonstrable tryptase activity.[6] The fibrin in the clotting tests was still solid after three days.

The close parallelism between tryptic and clotting activities, evident in these experiments, suggests an autocatalytic process of enzyme activation.

DISCUSSION

We should like to point out the undoubted confirmation, which Tagnon's[15] data afford, of the views expressed in frequent communications from the Michigan laboratories during the past three years. We have delayed full consideration of the role of serum tryptase in fibrinolysis because of a conviction that this is a minor and variable side-action of the role of the enzyme factor in blood-clotting. The present experiments with rabbit blood add emphasis to this view.

In evaluating the experiments of Eagle and Harris,[16] Tagnon is correct in pointing out that they compared crystalline trypsin to Ca + "thromboplastin" without attempting a definition of the latter term. Eagle's work must be credited with stimulating renewed interest in proteolytic enzymes and their relationships to blood clotting, although considerable older data appear in the literature and we must give a niche to the long-forgotten study of Collingwood and MacMahon, 1913,[17] suggesting a relationship of trypsin to progressive antithrombic action.

In extending Eagle's observations, Ferguson (op. cit.) has sought to demonstrate that the *natural* serum tryptase (long known to enzyme chemists) actually *is* the chief thromboplastic factor of the blood plasma. While it can act under conditions of not-too-great depression of the Ca-ionization as shown by Eagle,[16] Ferguson[3, 4] points out that Ca and cephalin are essential for optimal prothrombin activation. Particularly noted[3] in this connection is the "stability" of the clots obtained, which is a phenomenon again emphasized by Tagnon[15] in his rather obscurely presented view concerning "inhibitory" actions of "thromboplastin" (and "prothrombin"). Current studies of trypsin *inhibitors* are yielding very interesting data, which will appear from the Michigan laboratories in due course. That a deficiency of serum tryptase is the "bane of the Hapsburgs" is the Ferguson (1939)[9] theory of the causation of *hemophilia*.

SUMMARY

A new fibrinolytic method of protease (especially tryptase) assay is detailed. It can detect small differences within a range of (final) concentrations of 1:40,000 to 1:1,000,000 crystalline trypsin (1 mg. X. T. = 4.24×10^{-3} $[T.U.]_{Mb}$). While the wide fields of usefulness of the new methods are illustrated only at the extreme limit by the rabbit blood clotting experiments here pre-

sented, it is significant that this animal yields serum tryptase not in excess of 1:40,000 (X.T.) per cubic centimeter of materials tested and that appreciable quantities of enzyme liberation require coagulation(!) whether by added thrombin or by recalcification. With these relatively small amounts of enzyme (probably a species peculiarity), "thromboplastic" conversion of prothrombin to thrombin yields stable thrombin and stable fibrin clots.

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EFFECT OF TOURNIQUETS ON VENOUS BLOOD SUGAR VALUES*

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THE influence on the blood sugar level of a tourniquet employed to distend the veins in carrying out venepuncture has never been considered, and yet obvious possibilities exist.

METHODS

The present determinations were carried out, using a blood pressure cuff with mercury manometer attached as a tourniquet. A control series of observations was made without the use of a tourniquet. The tension applied to the upper arm was either above the systolic arterial pressure, "systolic tourniquet," or at or above the diastolic pressure, but distinctly below the systolic pressure, "diastolic tourniquet." Each one of the three procedures was carried out on five normal subjects in the fasting state. An interval of several days elapsed between each of the three experiments. The blood sugar determinations were carried out over a period of about six minutes.

The venous blood sugar estimations were made according to the method of Folin and Malmros[1] as modified for the photoelectric colorimeter by Saifer, Valenstein, and Hughes.[2] The determinations were carried out in duplicate on a carefully standardized photoelectric colorimeter.

TABLE I

CONTROL LEVELS OF BLOOD SUGAR MG. PER 100 C.C., CORRESPONDING TO CHART

	CASE 1	CASE 2	CASE 3	CASE 4	CASE 5
No Tourniquet	95	108	100	111	104
Arterial Tourniquet	88	98	84	102	89
Venous Tourniquet	81	93	99	125	103

RESULTS

The blood sugar series determined without a tourniquet serve as a control for the subsequent ones. The variations from the initial blood sugar in the 28 blood sugar estimations comprised in this group, varied above 6 mg. per 100 c.c. in only two instances: Case 2, 10 mg. below the control level, and Case 4, 10 mg. above the control level. From these results it may be concluded that changes of 10 mg. or less are within the limits of error for the present procedure.

The curves obtained when an arterial tourniquet was applied are distinctly more variable than when no tourniquet was used. In only three blood sugar estimations, however, did the changes exceed the 10 mg. limit set as the limit of error: 15 mg. per 100 c.c. deviation from the control in Case 2, and two values

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of 11 mg. in Case 3. It would seem justifiable to conclude that the arterial tourniquet effects no very significant changes in the venous blood sugar; the greater variability of the blood sugar with the arterial tourniquet, as compared with the venous blood sugar values when no tourniquet was applied, may be ascribed to the time required to inflate the cuff, when for a few seconds the venous tourniquet would be active and induce such variations as are discussed in the next paragraph.

The results with the venous tourniquet are distinctly different. They show changes from the control blood sugar varying from 18 to 24 mg. per 100 c.c. In no case was the variation less than 18 mg. per 100 c.c.; in four instances the blood sugar rose; in one the principal change was a drop; the most consistent fluctuation was a rise in the blood sugar followed by a drop.

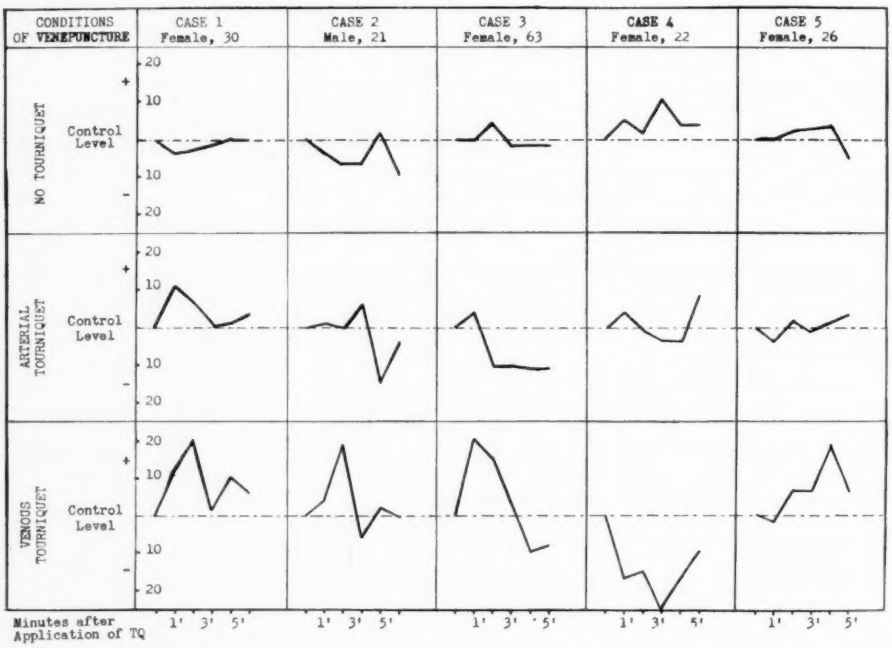

CHART I.—VARIATIONS OF THE VENOUS BLOOD SUGAR FROM THE CONTROL IN FASTING NORMAL SUBJECTS WITHOUT A TOURNIQUET, WITH AN ARTERIAL TOURNIQUET AND A VENOUS TOURNIQUET. RISE IN BLOOD SUGAR IS INDICATED BY THE HEIGHT OF THE CURVE ABOVE THE CONTROL LEVEL LINE, A DROP BY THE DEPTH OF THE CURVE BELOW THE CONTROL LEVEL LINE. THE CONTROL BLOOD SUGARS VARIED FROM 81 TO 125 MG. PER 100 C.C. SEE TABLE I.

DISCUSSION

Without a tourniquet the venous blood sugar level, obtained at minute intervals over a six-minute period, shows no variations that cannot be ascribed to the limit of error inherent in the procedure employed. The results after the application of a venous tourniquet indicate that some adjustments occur that will cause the blood sugar to undergo fluctuations that are significant from the clinical point of view. Such changes deviate about 20 mg. per 100 c.c. from the expected value; they generally take place within two minutes after the application of the venous tourniquet. As a rule there is a rise of the blood sugar,

though in one instance (Case 4) there was a marked fall. These initial variations in the venous blood sugar are transient, and are followed by a shift in the opposite direction after the tourniquet has been in effect two or three minutes.

The increase of the glucose concentration in the venous blood while a venous tourniquet is acting may be ascribed to the forcing of arterial blood with its higher sugar content into the occluded veins.

Ebert and Stead[3] have shown that one of the effects of venous tourniquets on the extremities is a reduction of the plasma volume in the blood vessels, resulting from a transudation of fluid into the surrounding tissues. Since the concentration of glucose is greater in the plasma than in whole blood, such a diminution of plasma volume would account for a lowering of the blood sugar when the amount of plasma was less and the red blood cell content was greater.

These two factors, increased accumulation of arterial blood in the veins and diminished plasma volume, occurring at different intervals after the application of a venous tourniquet, would account for either a rise or a drop in the venous blood sugar. The absence of these two influences with no tourniquet at all would explain the stabilization of the venous blood sugar under those circumstances.

Venous blood sugars determined after the application of an arterial tourniquet are slightly more variable than those when no tourniquet is used. This may be accounted for by the transient phase of venous tourniquet pressure in applying the arterial tourniquet. It is probable that if the tourniquet would be adjusted with great speed, the venous blood sugar figures derived from the use of the arterial tourniquet would duplicate those obtained without a tourniquet.

CONCLUSIONS

Without a tourniquet, the venous blood sugar levels remain constant over a period of five minutes. The application of a venous tourniquet results immediately in significant changes in the venous blood sugar amounting to 20 or even 25 mg. per 100 c.c. as either an increase or a diminution from the control value. The use of an arterial tourniquet causes some variations in the venous blood sugar, which, however, are much less marked than those obtained with the venous tourniquet. For the greatest accuracy a tourniquet should not be used in obtaining blood for venous blood sugar determinations.

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AN APPARATUS FOR STORAGE OF GAS-FREE SOLUTIONS FOR USE WITH THE VAN SLYKE GASOMETRIC METHODS*

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VARIOUS apparatus have been designed to keep the reagents used for gasometric analysis gas-free for some length of time. Most of them, however, as pointed out by Guest and Holmes,[1] have the disadvantage that the alkaline solutions have to pass a glass stopcock,[2, 3] the grease of which does not

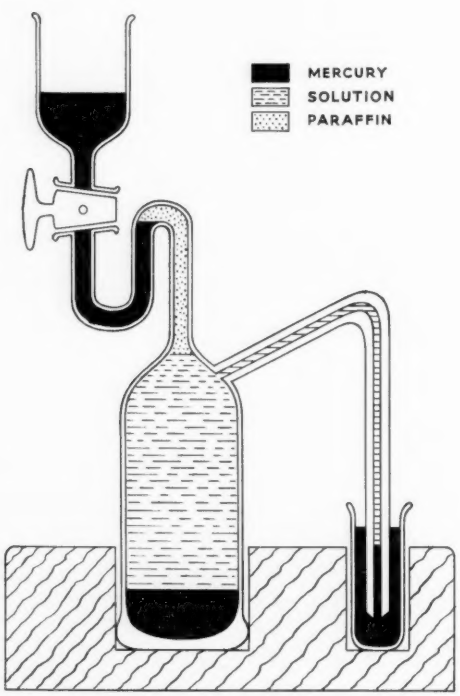

Fig. 1.

hold long against the alkaline solutions and leakages occur easily. The apparatus of Holmes[4] eliminates this difficulty, but it has the disadvantage of having to be made by an expert glass blower.

The following apparatus has been designed to take the place of more complicated pipettes; it has at the same time the advantage that only mercury or paraffin comes into contact with the stopcock.

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The apparatus was blown for us by Mr. V. J. Hinkley.
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As seen in Fig. 1, the vessel consists mainly of four parts: (1) the upper storage chamber for the mercury, (2) the lower storage chamber for the solution, (3) an S-shaped tube connecting both vessels and provided with a stopcock, and (4) a delivery tube which is immersed into a small test tube filled with mercury, providing the seal.

Filling of the Apparatus.—Before filling the vessel with the solution, it is filled with liquid paraffin, in which process special care has to be taken that the paraffin does not contain any air bubbles. Following this, the paraffin is displaced by the solution in the manner indicated in Fig. 2. The delivery tube is fitted into the bottom of the cup of the Van Slyke, a small rubber ring making

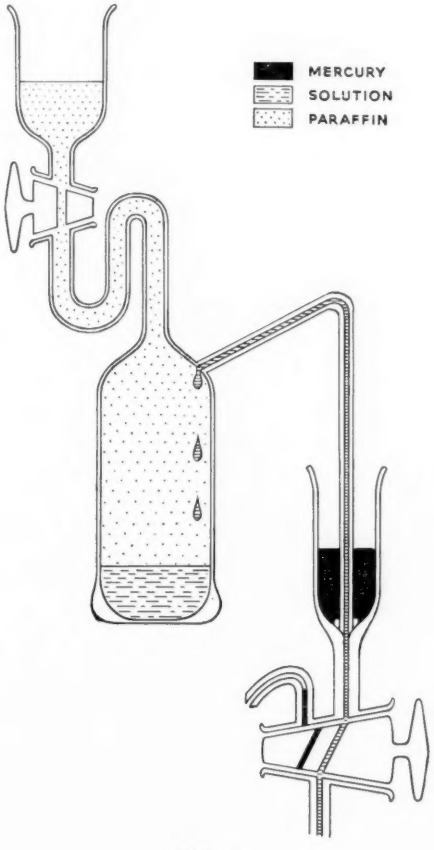

Fig. 2.

the connection airtight, and mercury sealing the connection as well. With the stopcock of our vessel open, and the leveling bulb of the Van Slyke in the upper ring, the upper cock of the Van Slyke pipette is opened and the gas-free solution is forced slowly up the delivery tube of our vessel. It then drops through the paraffin to the bottom of the storage chamber, displacing the liquid paraffin into the upper chamber of the vessel. The solution is thus collected in the storage chamber without coming into contact with the air. When all the solution has been delivered to the storage vessel, the stopcock of the vessel is closed, and the paraffin in the upper vessel is recovered. About 10 c.c. of mercury are

then poured into the upper chamber; part of it is allowed to fill the S-shaped connection, providing a seal. The vessel may then be placed in a convenient stand, its delivery tube being immersed into mercury as shown in Fig. 1. This provides a complete seal, and solutions have been stored gas-free in this way by us for many months.

The Use of the Apparatus.—In order to get the solution into the Van Slyke during an analysis, the upper storage chamber is filled with sufficient mercury. On opening of the stopcock, the mercury by its own gravity will flow from the upper into the lower storage chamber, displacing the solution via the delivery tube. After closing of the stopcock and after returning of the vessel into the stand, complete sealing is again obtained. Mercury has to be added from time to time in the upper vessel, as it is used for replacing the solution.

The dimensions of the apparatus can be clearly seen from the accompanying figures. Actually, any size for the vessel storing the solution is possible, but for practical purposes 100 c.c. capacity for the chamber holding the solution was found most suitable. The upper (mercury) chamber should conveniently hold about 30 c.c. in order to take the paraffin which is being displaced when filling, since 25 c.c. of solution may be made air-free at one time with the Van Slyke. The delivery tube is made of capillary tubing having a bore of 1.5 mm. It is constructed in such a way that the vertical part of it during filling and delivery of the solution is sufficiently far away from the cup of the Van Slyke as to allow smooth operation of the latter's stopcock.

SUMMARY

A self-sealing apparatus for the storage of gas-free solutions is described, which has the following advantages:

1. It eliminates the use of rubber parts and of glass stopcocks where the grease could be dissolved by the alkaline solution stored in the vessel.

2. It can be easily filled.

3. It provides a mercury seal for the delivery tube.

4. The apparatus is characterized by its simplicity and therefore can be easily made by a moderately experienced glass blower. It is much less liable to break than the more complicated vessels in use.

The vessel has been found most convenient, especially under such conditions where gasometric analyses are undertaken only at long intervals.

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A RAPID MICROANALYTICAL METHOD FOR LEAD IN URINE*

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A METHOD is herein described for the rapid microanalysis of lead in urine. Aub and associates[1] mention the fact that ammonium hydroxide quantitatively precipitates lead as lead phosphate from urine. If such be the case, this would afford an excellent means of separating the lead from interfering organic matter in urine. Experimentation,[2] however, showed that excess ammonia did not result in a 100 per cent recovery. Fairhall[3] states that there is a 100 per cent precipitation of lead with ammonia at pH 10. This was found to be experimentally correct.

Several excellent procedures have been described for the destruction of organic matter in urine in preparation of the sample for the determination of lead. These employ either the dry muffle heat below 500° C. or the various acid oxidation modifications. Although fairly rapid, these methods are not as rapid as the phosphate precipitation. Moreover, in the wet oxidation methods, the quantities of urine analyzed have been so reduced that slight deviations become appreciable and significant when calculated on a liter or per diem basis. Larger samples of urine may, of course, be digested, but then there is time consumption in concentrating the volume.

PRINCIPLE OF METHOD

The method is based upon the total precipitation of lead by the addition of ammonium hydroxide to urine. Lead phosphate is very insoluble and is easily filtered out along with the alkaline earth phosphates of calcium and magnesium. This precipitate is subsequently dissolved with hot dilute nitric acid and is heated for a short time to oxidize any adhering organic material. Citric acid is added to keep the phosphates in solution during later neutralization, and potassium cyanide is added to form complex ions with interfering metals.** When the pH is adjusted between 9 and 10, the lead is extracted with dithizone†† to separate it from extraneous salts and interfering substances. Once the lead is separated, it is then split from the dithizone and titrated with standardized dithizone for an accurate determination. Under the above conditions only bismuth, tin, thallium, and lead react with dithizone. To remove the interference of bismuth and tin, these are previously extracted with dithizone while the solution is yet acidic. Lead is not extracted in this acid solution. Thallium, as a source of interference, has not been removed, but its occurrence is rare and unlikely.

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††Dithizone is the abbreviated name for diphenylthiocarbazone.

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REAGENTS

All solutions should be kept in pyrex glass bottles. Water is distilled from an all-pyrex still. Ammonia water and nitric acid should have low lead content.

Potassium Cyanide Solution: 10 Gm. of potassium cyanide (low in lead) dissolved in water to make 100 ml.

Citric Acid Solution: 200 Gm. of citric acid (low in lead) dissolved in water to make 500 ml.

Standard Lead Solution: 160 mg. of lead nitrate dissolved in 1,000 ml. of water. One milliliter is equivalent to 100 gamma* of lead.

Dithizone 1 for Preliminary Extraction: 25 mg. of dithizone dissolved in 400 c.c. of chloroform.

Dithizone 2 for Titration: 100 ml. of dithizone 1 diluted with 100 ml. of chloroform. This is standardized against known lead. Approximate value is 1 c.c. = 5 gamma lead. If kept in a brown bottle and in a cool place, the titer will not change for several months. This titer is satisfactory for amounts of lead near normal values. However, if larger amounts of lead are expected (this is determined approximately by the amount of dithizone required for the preliminary extraction), a stronger titer may be prepared. Dithizone 1, when standardized, is approximately 1 c.c. = 10 gamma lead. The titer should be calculated to at least one decimal unit.

All glassware should be of pyrex quality.

METHOD IN DETAIL

Description.—The total twenty-four-hour specimen of urine is collected and the volume measured. One-third of this volume is taken for analysis. A few drops of phenolphthalein and then dropwise ammonium hydroxide are added until the urine turns pink (pH 9 to 10). This is well shaken and stoppered and the precipitate which forms is allowed to settle for about thirty minutes. The precipitate is then separated by being filtered through a Buchner funnel with suction and refiltered if necessary. The precipitate is washed with several small portions of alcohol. The filter paper is then inverted so that the precipitate now faces the perforations of the funnel. Approximately 30 to 50 ml. of hot 3 per cent nitric acid are successively poured over the precipitate until it either dissolves or is completely transferred with the filtrate to the flask. This is more easily accomplished without suction. The total redissolved precipitate is then gently boiled for approximately twenty minutes. It is adjusted to a pH of from 3 to 3.5 (lower range of methyl orange) with the dropwise addition

*Gamma = 0.001 mg.

of ammonium hydroxide and transferred to a Squibb separatory funnel. It is then shaken out with 5 to 10 ml. of dithizone 1, and the dithizone-chloroform layer is discarded. This extracts and eliminates the possibility of either tin or bismuth appearing later as an interference. Lead is not extracted at this pH. Then 15 ml. of citric acid are added and the solution neutralized with ammonium hydroxide. With the addition of 2 ml. of potassium cyanide, the solution is ready for the lead extraction. In an ammoniacal solution and in the presence of potassium cyanide, dithizone has a selective affinity for lead, forming a cherry red complex of lead dithizone which is soluble in chloroform.

A small quantity of dithizone is run into the solution from a burette and vigorously shaken. The rapid appearance of a cherry red chloroform layer indicates the presence of lead. After each addition of dithizone, vigorous shaking is necessary and the colored chloroform layer is separated carefully and transferred to another separatory funnel. The last traces of lead dithizone are washed through with several small portions of chloroform. These are also collected and saved. The proximity of the end point (complete removal of lead) may be roughly estimated by the diminution of intensity of the cherry red color. If it is very red, a larger amount of dithizone may be added and again shaken and separated. After each shaking, the funnel is inverted and the stopcock opened to relieve the pressure. This is continued until all the lead has been extracted, which is indicated when further addition of dithizone does not impart a cherry red color to the chloroform layer, but on vigorous shaking a green color persists due to excess dithizone. The presence of many extraneous salts prevent a sharp end point (cherry red to green). Therefore, the first titration-extraction is not a reliable indication of the quantity of lead present. The end point is difficult to see because of the lag of the transition of colors (cherry red, pink, violet, blue, green). To rectify this, the several portions of extracted lead dithizone should be carefully collected and saved. These combined chloroform extracts are shaken with 15 ml. of 1 per cent nitric acid which quantitatively separates the lead into the aqueous solution. The green chloroform layer is discarded. The aqueous layer is washed by shaking with a fresh portion of chloroform to remove any residual dithizone. To the solution are added several drops of phenolphthalein and dropwise ammonia until alkaline, then 2 ml. of potassium cyanide, and lastly dropwise citric acid until the solution is just faintly pink. If the end point is overrun, a drop of dilute ammonium hydroxide will adjust it.

The solution is now titrated with standard dithizone 2. The dithizone may be added in one large portion to within 5 ml. of the total amount required in the first extraction (when cherry red color started to change). The separatory funnel is thoroughly shaken, allowed to stand until the layers separate, and the red chloroform layer is then removed. From this point, the titration continues carefully. The dithizone is delivered in successive small quantities (approximately 0.5 ml. portions), and it is necessary before each shaking to add approximately 1 ml. of chloroform so as to have enough volume to see the color. After each addition, the solution should be vigorously shaken, allowed to settle, and then the chloroform layer run out and discarded. This is continued until the end point is reached, which is a persisting light green color in the chloroform layer.

For standardization a known lead solution is titrated with the dithizone solution using the procedure previously described. Since the lead is in pure solution, the preliminary titration-extraction is not necessary. The quantity of lead in the standard used, divided by the number of milliliters of dithizone solution required, represents the dithizone lead equivalence.

A lead blank is determined for the reagents. However, since the reagents should be of highest quality or are previously deleaded, the blanks should be relatively low.

TABLE I

ANALYSIS OF 500 ML. PORTIONS OF POOLED URINE

LEAD ADDED (GAMMA)	TOTAL LEAD RECOVERED (GAMMA)	EVALUATION OF THE AMOUNT OF LEAD ADDED (GAMMA)
00	23	--
00	22	--
25	47	25
25	47	25
50	73	51
50	70	48
100	118	96
100	123	101
150	169	147
150	172	150
200	212	190
200	224	202
500	505	483

Table I indicates that this method for lead is applicable to urine, using dithizone as a standard solution. The maximum error was ±5 per cent.

SUMMARY

1. Excess ammonium hydroxide as prescribed by Aub and associates for the precipitation and absorption of lead is unsatisfactory.

2. The lead may be totally separated from urine at pH 10 as suggested by Fairhall.

3. The lead is quantitatively determined by an extraction-titration procedure with dithizone reagent.

4. This method for determining lead in urine is rapid, sensitive, and reliable, and the maximal deviation is ± 5 per cent.

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